OVEREXPRESSION OF TOBACCO OSMOTIN PROTEIN IN CARROT (*Daucus carota* L.) TO ENHANCE DROUGHT TOLERANCE

A Thesis

by

ALI HANI HAMZA ANNON

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Approved by:

Co-Chairs of Committee,	Kevin M. Crosby
	Keerti S. Rathore
Committee Member,	Leonardo Lombardini
Head of Department	Dan Lineberger

December 2012

Major Subject: Horticulture

ABSTRACT

Lack of water is one of the most significant issues that already threaten world agriculture as many countries are unable to meet the demand for water to grow the crops. To make matters worse, the water availability is expected to fall by half by 2050, thus severely restricting agriculture production. Genetic engineering of crops to enhance their tolerance to such unfavorable environment represents one of the few approaches that can help us address this problem. Osmotin and osmotin-like proteins are stress proteins, belonging to the plant PR-5 group of proteins, which induced in response to various types of biotic and abiotic stresses in several plant species. Carrot plants were transformed with tobacco osmotin gene that encodes a protein lacking 20 amino-acid sequence at the C terminal end under the control of CaMV 35S promoter using the *Agrobacterium*-mediated transformation method. The gene integration and expression were confirmed by Southern and Western blot analyses and the transgenic plants were evaluated for their ability to tolerate drought stress. Under drought conditions, transformants exhibited slower rates of wilting compared to the wild-type and gained the ability to recover faster than their untransformed counterparts when the drought stress was alleviated. Under water stress, transformants showed lower levels of H_2O_2 accumulation, reduced lipid peroxidation and electrolyte leakage, and higher leaf water content. Taken together with some earlier reports, our results provide additional evidence for the protective ability of tobacco osmotin protein against drought stress and suggest a possible means to achieve tolerance against a serious type of abiotic stress.

DEDICATION

To my dearest father and my lovely mother.

ACKNOWLEDGEMENTS

I would like to thank my advisor, Dr. Kevin Crosby for his guidance, supervision and support during my courses and research. Dr. Crosby was an easy going person with a friendly characteristic and I never felt uncomfortable sharing my thoughts and ideas with him. I am also sincerely thankful to Dr. Keerti Rathore for his support and advice during my research work. He was really my backbone and the man who taught me almost everything about genetic transformation and I wish to have at least half of his genius mind in the future. I also want to thank Dr. Leonardo Lombardini for his support and lovely personality.

My special thanks are to my awesome lab members, LeAnn Campbell, Loren Tollack, Dr.Sreenath Palle, Dr.Sameer Joshi, Dr.Devendra Pandeya, and Jazmina Urriola, who were not only lab mates, but as brothers and sisters to me. They helped me a lot during my research and exhibited as much compassion, friendship and courtesy as one can expect. I would like to extend my gratitude to my sponsor, The Norman Borlaug Institute for believing in me and for their support and encouragement throughout my study specially Dr. Maad Mohammed and Eric Brenner. I would also like to thank my friends and colleagues and the department faculty and staff for making my time at Texas A&M University a great experience.

Finally, my grateful thanks go to my very gentle and ideal father Hajji Hani, my lovely and kind mother Hajja Sahar, my beautiful and supportive wife Shahad, and my dearest brothers Shamil and Ammar for their love and patience during my master's study.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

1. INTRODUCTION

The impact of drought

Drought is one of the most serious issues that threaten global agriculture, which can happen recurrently in many climates around the world. Scientists define drought as a lack of moisture caused by a natural reduction in the amount of precipitation over a period of time associated with increased water demand that exceeds the natural water resources (Wilhite and Buchanan-Smith, 2005; Wilhite and Glantz, 1985). The consequences of drought impact heavily the global economy, as well as the environment. Bryant (1991) ranked drought as number one among all natural hazards, depending on several characteristics including economic loss, severity, duration, loss of life, and social effect. Sub-humid, semiarid, and arid regions are frequently subjected to drought due to their highly variable inter-annual precipitation. Consequently, agriculture in these regions is often tenuous and it gets more vulnerable in the below-normal precipitation years. Each drought episode is unique in its climatic characteristics because it is often related to timing (delay in the start of the rainy season, infrequent of rains during principle crop growth stages), and the intensity and number of rainfall events (Sivakumar et al., 2011).

Sivakumar et al. (2011) listed several characteristics that can distinguish drought from other natural hazards in many ways depending on the severity and duration of its impact. They also stated that the parameters that define drought are different due to the

variability in the types of droughts that exist. For example, meteorological drought is defined by the decrease in precipitation over an extended period of time, while agricultural drought can best be defined as the lack of soil moisture that influences the crop production potential. Additionally, hydrological drought is best defined by deficiencies in surface and subsurface water supply. Many countries have been facing severe drought conditions since the recording of climate data. These countries have experienced less and inconsistent rainy seasons, deficiencies in water storage capacity, and irrigation water shortages that have threatened their agricultural sectors. The Murray-Darling River Basin in Australia is a clear example of drought consequences. In the last two decades, this river has been subjected to periods of prolonged droughts and in 2008, the river water level was less than a quarter of its long-term average. Moreover, southwestern regions of China are experiencing the worst drought in six decades which forced more that 2 million people back into poverty (Sivakumar et al., 2011). As for the Middle East and the Horn of Africa, where most of their lands are located within the arid and semi-arid ecozones, drought is often considered frequent phenomenon. Hazell et al. (2001) classified the regions of the Middle East and North Africa into six bioclimatic zones based on the annual rainfall (Table1).

Table 1: Classification of Mediterranean Bioclimatic Zones in Middle East by Precipitation (Hazell et al. 2001)

Zone type	**Annual rainfall (mm)**	**Percent of arable area**
Super- arid	< 200	0
Semi-arid 1	200 – 400	74
Semi-arid 2	400 – 600	14
Sub-humid	600 – 800	10
Humid	800 – 1200	1
Super-humid	> 1200	1

The United Nations Development Programme (UNDP) reported that many countries within the Middle East region including Iraq, Syria, Turkey, and Iran have been facing severe drought conditions since the beginning of the current century. Fig. 1 shows UNDP (2011) declared affected areas in the Middle East under drought (Fig.1).

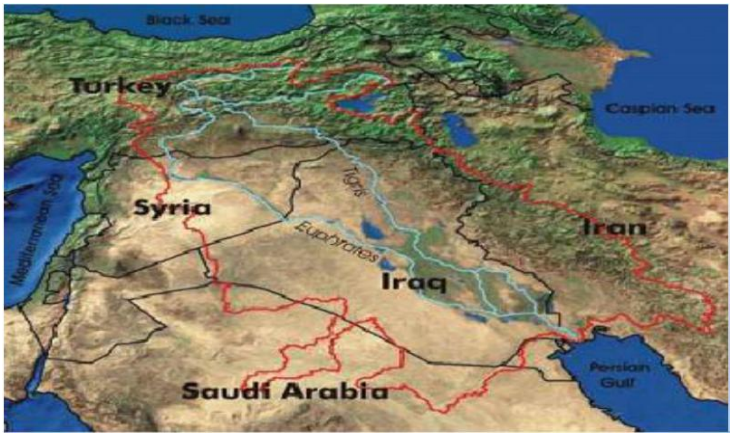


Figure1: A recent map of the Middle East that shows the wide spread drought (outlined in red; UNDP, 2011)

The drought situation gripping many parts of the world has severely impacted the water resources, livestock, and agriculture and threatens a possible famine. Genetic modification of crops for drought tolerance represents one of many approaches that can help alleviate low agricultural productivity caused by inadequate water supply.

Carrots general introduction

Carrot (*Daucus carota* L.) is a diploid species (2n=2x=18) and a member of the genus *Daucus*, in the Apiaceae family. The genus *Daucus* contains approximately 22 to 25 species (Saenz, 1981) which grow commonly in the Mediterranean region and Southwest Asia. Moreover, some representative species are found in North Africa, Australia, New Zealand, and the American continent (Peterson and Simon, 1986). Afghanistan and Central Asia are considered to be the primary center of genetic diversity of carrots, whereas the Anatolian region of Asia Minor is considered as the primary center of origin for Western carrot (Heywood, 1983; Simon, 2000).

Carrot is among the top ten, most economically important vegetable crops in the world, both in terms of production and market value (Rubatzky et al., 1999; Simon, 2000; Fontes and Vilela, 2003; Vilela, 2004). World-wide production of carrots and turnips in 2010 was estimated at 33.66 million metric tons, while their area harvested, yield, and seed production were 1.164 million ha, 0.289 million/ha, and 18186 tons, respectively (Food and Agriculture Organization (FAO), 2010). Carrot is also widely used in Iraq with a total production of 27,636 tons on 2,175 hectares in 2010 (FAO, 2010).

Although carrot is a biennial species, it can be handled as an annual crop if roots are replanted for seed production after storage at 2-5°C for 6-8 weeks (Peterson and Simon, 1986). After vernalization, carrot develops perfect flowers, each one consisting of five sepals, petals, and anthers and split pistil. Carrot flowers contain two locules and each one contains a functional ovule. Carrot fruits are schizocarps consisting of two mericarps, and each one of them is an achene (Rubatzky et al., 1999). These flowers are protandrous thus promoting cross pollination, although selfing can occur between umbels at different developmental stages. Flowers are grouped in umbellets and arranged in primary, secondary, tertiary, and quaternary umbels. Each flower can produce two seeds leading to more than 5000 seeds per plant (Simon, 2000). Apiaceae species are characterized by having unique compounds like umbelliferose, petroselinic acid, and polyacetylenes. They also have specific phenols, phenylpropanoids, terpenes, saponins, and coumarins in roots, leaves or fruits (Hegnauer, 1990). These secondary compounds in Apiaceae taxa are well-recognized in traditional and modern medicines.

Carrots are also rich in carotenoids, which provide vitamin A in the human diet (Simpson, 1983). Vitamin A deficiency causes xerophthalmia, night-blindness, and even death affecting millions of children each year, especially in developing countries (Simon, 1997). Stavric (1994) described beneficial effects of carrot flavonoids and carotenoids on the cardiovascular system, as well as their anticarcinogenic properties. Like most of the vegetables from the Apiaceae family, carrot is a cool-season crop (Rubatzky et al., 1999). Carrots require 12-14 weeks for commercial production and are usually grown in the temperate climates, although it can be produced during winter in

subtropical regions, and in high-elevation tropical climates. The optimal growth temperature for carrots is between 15 and 21°C and temperatures less than 10°C or more than 25°C reduce organoleptic properties and limits the production. The availability of appropriate moisture throughout the growing season is a crucial production requirement. Although carrot's water need is moderate, the species cannot be considered drought resistant (Rubatzky et al., 1999).

The utilization of stress proteins

Plant adaptation to environmental stresses relies upon the triggering of certain molecular pathways involving stress perception, signal transduction, and the expression of specific stress-related genes and metabolites (Vinocur and Altman, 2005). Several proteins shown to be synthesized in response to an altered environment and have been reported in plants as stress or shock proteins including HSPs, SRPs, TLPs, and many others (Henrik and Kees, 1972; Amar and Reinhold, 1973; Barnetr et al., 1980; Cooper and Ho, 1983; Diaz de Leon et al., 1980; Duque, 2011). However, only a few of these proteins are actually involved in known metabolic or physiological processes (Hanson et al., 1984; Sachs et al., 1980). Most of these proteins are synthesized as an immediate response to an altered environment including anoxia, temperature, wounding, and osmotic stress (Singh et al., 1985). Several proteins have been shown to be induced by hyperosmotic (low osmotic potential) environments (Gisbert et al., 2000). One of these proteins is osmotin, a unique protein belonging to a group of pathogensis-related

proteins (PR-5) family (Singh et al., 1987, 1989; Bol et al., 1990; Zhu et al., 1993, 1995). The protein was first isolated in 1985 from the cultured cell of tobacco adapted to relatively high concentration of NaCl or polyethylene glycol (PEG). The protein was named osmotin due to its induction by low water potential of the growth medium (Singh et al., 1985, 1987). Osmotin belongs to a group of cationic proteins that exists in two forms with slightly different molecular weights: osmotin I with an isoelectric point (pI) of 8.2 or greater that is readily soluble in standard buffers; osmotin II with a pI of 7.8 that is soluble only with detergents and urea (Singh et al., 1985, 1987). The protein is first synthesized as a preprotein with a molecular weight of 26.380 KDa which then matures to 23.984 kDa size (Singh et al., 1989). Osmotin has peptide sequence similar to many stress-related proteins such as the tomato protein NP24, the bifunctional trypsin/α-amylase inhibitor from maize, the tobacco antiviral protein gp22, the potato PR protein C, and the sweet protein thaumatin (Bryngelsson and Green, 1989; Edelbaum et al., 1990; King et al., 1988; Pierpoint et al., 1990; Richardson et al., 1987; Singh et al., 1987). Thaumatin is a sweet protein present in the fruits of a tropical plant *Thaumatococcus daniellii* (Henrik and Kees, 1972). It has an amino acid sequence that is significantly homologous with osmotin, along with several common features including similar molecular weight, the lack of sulfhydryl residues and the presence of several disulfides, a high proportion of proline, and a basic pI. The sweet test of Thaumatin is basically due to high lysine content and certain lysine residues, however, it differ from osmotin that has lower lysine residues (Singh et al., 1987). The same group (Singh et al., 1985, 1987) also showed that osmotin protein can be induced in response to treatment

with abscisic acid (ABA) or NaCl and PEG-mediated water stress. However, salinity or osmotic stress did not induce osmotin transcripts in an ABA-deficient mutant of tomato, suggesting the involvement of ABA in osmotin mRNA accumulation (Grillo et al., 1995). In addition to induction by ABA, osmotin transcription is controlled by a wide range of tissue specific factors and various hormonal signals indicating considerable transcriptional and post-transcriptional regulation of osmotin mRNA (Kononowicz et al., 1992; LaRosa et al., 1992; Raghothama et al., 1993). Some studies reported that the synthesis and accumulation of osmotin mRNA is regulated by at least six hormonal or environmental signals including ABA, ethylene, tobacco mosaic virus infection, salinity, desiccation, and wounding in both cultured cells and whole plants of tobacco (LaRosa et al., 1987,1992; Singh et al., 1987). Neale et al. (1990) reported that osmotin and other PR protein genes were overexpressed in tobacco tissue treated with cytokinin that resulted in the induction of flowering. In addition, even in intact plants, osmotin is expressed in floral organs during their development. These results indicate a role for osmotin and other PR genes during transition from the vegetative to reproductive state. Much of the secreted osmotin protein was found to be localized in electron-dense inclusion in the vacuoles. It was barely detectable in the ground cytoplasm with no preferential localization within the cytoplasm. It has also been detected in the extracellular matrix of plant cell, although it might be just an acidic form of the protein as are other extracellular PR proteins (Casas et al., 1992). Kononowicz et al. (1992) demonstrated the spatial and temporal pattern of the osmotin promoter during normal plant development and after adaptation to NaCl. They found that the osmotin promoter

exhibits a high level of activity in mature desiccated pollen grains during anther dehiscence and in pericarp tissue at the final desiccating stages of fruit development. They also found that there are temporal and spatial pattern similarities between Na^+ and K^+ distribution within whole plants and the osmotin promoter activity; for example, an increase in the promoter activity in the root elongation zone, stem parenchyma, and older leaves. The authors state that these similarities support the hypothesis that the expression of the osmotin gene is an adaptive response of plants to osmotic stress.

Considerable numbers of studies have reported the protective role of osmotin against biotic and abiotic stresses. Overexpression of the osmotin gene in tobacco plants improved their tolerance to salinity and drought stress (Barthakur et al., 2001). Along with tobacco, the constitutive expression of the osmotin gene conferred tolerance against biotic and abiotic stresses in wheat (Noori and Sokhansanj, 2008), strawberry (Husaini and Abdin, 2008), cotton (Parkhi et al., 2009), and mulberry (Das et al., 2010). In their work with olive trees, Angeli and Altamura (2007) speculated that osmotin can play a role in cold acclimation by its involvement in programmed cell death, blocking cold-induced calcium signaling, and affecting cold-induced cytoskeleton alteration. The osmotin protein plays a bifunctional role in osmotic stress adaptation and plant defense (Kononowicz et al., 1992; La Rosa et al., 1992; Nelson et al., 1992; Zhu et al., 1995). Several hypotheses have been proposed suggesting osmotin's mode of action, either by facilitating the confinement of solutes in the vacuoles (Barthakur et al., 2001), or by its involvement in altering the plant structure and metabolism during the osmotic adjustment (Singh et al., 1987). It is also believed to protect the proteins' native structure

and repair denatured proteins during stress (Evers et al., 1999). However, the actual protective mechanisms of osmotin against abiotic stress are still not very clear and are under investigation.

In addition to osmotic stress, osmotin has been shown to be induced in response to viral and fungal infections in tobacco and tomato (Stintzi et al., 1991; Woloshuk et al., 1991). Overexpression of the osmotin gene in transgenic potato increased tolerance to late blight caused by *Phytophthora infestans* and delayed the development of the disease symptoms (Liu et al., 1994). Tobacco osmotin was also shown to inhibit the growth of *Candida albicans, Neurospora crassa,* and *Trichoderma reesei* (Vigers et al., 1992). In a large-scale *in vitro* study, Abad et al. (1996) demonstrated the antifungal properties of osmotin against a wide range of fungal pathogens. They showed that osmotin can cause membrane permeabilization and deplete the pH gradients across the cell wall of sensitive pathogen species. However, different factors can affect the sensitivity to osmotin including the growth stage of the fungus, the concentration of NaCl and other inorganic cations, and the cell wall properties.

The reports described above suggest that utilization of tobacco osmotin gene in carrot plants might be a possible approach to produce transgenic plants that can withstand the reduction in water availability. Therefore, carrot plants were transformed with the tobacco osmotin gene under the control of the Cauliflower Mosaic Virus (CaMV35S) promoter and terminator, using the *Agrobacterium tumefaciens* transformation method. Our aim was to obtain carrot lines that constitutively express the tobacco osmotin and evaluate their ability to tolerate drought stress. During the course of investigation, these

carrot lines were subjected to water deficit conditions and several physiological, biochemical, and developmental parameters were examined.

2. MATERIALS AND METHODS

Plant materials and tissue culture

Approximately 100 carrot (*Daucus carota* L. var. Chantenay; Burpee Garden products Co., Warminster, PA) seeds were surface sterilized in 70% ethanol for 30 s followed by three washes with sterile distilled water. The seeds were then soaked in 15% sodium hypochlorite and a drop of Tween-20 for 10 min with continuous shaking. After four washes with sterile distilled water, the seeds were allowed to germinate on the MS medium (Murashige and Skoog, 1962) for several days. Hypocotyl explants from germinated seedlings were grown on callus induction medium (CS1) for 1-2 months according to the protocol described by Sung et al. (1981) except that 2,4-dichlorophenoxyacetic acid (2,4-D) was used at 1 mg/L instead of 0.1 mg/L concentration (Pawlicki et al. 1992).

Agrobacterium-mediated transformation of carrot callus

Carrot transformation was performed following the protocol established by Sunilkumar and Rathore (2001) and Rathore et al. (2006) using the *Agrobacterium* System. Briefly, *A. tumefaciens*, strain LBA4044 [harboring the binary vector pCAMBIA2300-Osmotin (Parkhi et al. 2009)] cultures were grown for 8 h in lysogeny broth (LB) medium [10 g/L tryptone (Difco Laboratories Inc., Sparks, MD), 5 g/L yeast extract (Difco Laboratories Inc.), 5 g/L NaCl, pH 7.0] containing 10 mg/L rifampicin

and 100 mg/L kanamycin. The suspension was then centrifuged at 4000 rpm for 20 min at 4°C. The collected cells were re-suspended in 10 mL modified autoinducer bioassay (AB) minimal medium [(5 g/L glucose, 50 mL/L AB salts (Chilton et al., 1974), 2.5 mM sodium potassium buffer, 3.9 g/L 2-(*N*-morpholino)ethanesulfonic acid (MES) (Sigma-Aldrich, St Louis, MO), pH 5.5)] and shaken overnight at 28°C. The suspension was centrifuged again and the pellet was re-suspended in 10 mL pre-induction medium (PIM) (10 g/L glucose, 14.62 g/L MES, 0.1 M sodium phosphate buffer, 50 mL/L AB salts, pH 5.6) containing 20 µL acetosyringone (10 mg/mL). For approximately every 100 mg-size of callus, 10 µL of *A. tumefaciens* suspension was applied. After 3 d of co-cultivation on CS1 medium containing 20 mg/L acetosyringone, the calli were transferred to fresh CS1 medium containing 500 mg/L cefotaxime and 100 mg/L kanamycin to eliminate *Agrobacterium* and to select transformed cells. After two months, 2,4-D was removed from the selection medium and the calli were maintained on this medium until the initiation of somatic embryos.

Regeneration of transformed calli

Somatic embryos that developed were kept on CS1 medium that was devoid of both 2,4-D and antibiotics in Magenta boxes at room temperature with 16/8 h photoperiod. After the plantlets reached an appropriate size, they were transferred to 8.9 cm pots containing Sunshine #5 soil medium (SunGro Horticulture, Seba Beach, Canada). The transgenic plants were grown under a 16/8 h photoperiod regime with

20°C/15°C day/night temperatures and 35% humidity settings in the growth chamber (Rubatzky et al., 1999). Afterwards, these transgenic plants were transferred to 3.78 L pots and grown under similar conditions.

Flower initiation and seed production

Following the vegetative development, carrot taproots were harvested and carefully cleaned with tap water to remove soil particles. Each taproot was treated with 1 g/L fungicide (Banrot, Scotts-Sierra Crop Protection Company, Marysville, OH), separated individually in plastic bags, and incubated at 4-6°C for 2 months of vernalization treatment to induce flowering. Vernalized roots were replanted in 3.78 L pots and monitored for the initiation of flower stalks. After the plants flowered, each individual transgenic line was pollinated individually by hand to maintain self-pollination using a small brush designated for each plant. The seeds were harvested when the seed stalk turned brown and kept at 4°C in sealed plastic bags.

Osmotin construct

A truncated tobacco osmotin gene lacking the sequence encoding a 20 amino acid-section at the C-terminal end (Singh et al., 1989; Liu et al., 1996) was ligated into the binary vector pRTL2 (Restrepo et al., 1990; Fig.2). The expression cassette contained the CaMV 35S promoter with double enhancer, a translational enhancer from

tobacco etch virus (TEV), the osmotin gene, and the CaMV 35S terminator. The cassette was isolated and ligated to pCAMBIA2300 to create the plant transformation vector pCAMBIA2300-Osmotin (Parkhi et al., 2009; Fig.3).

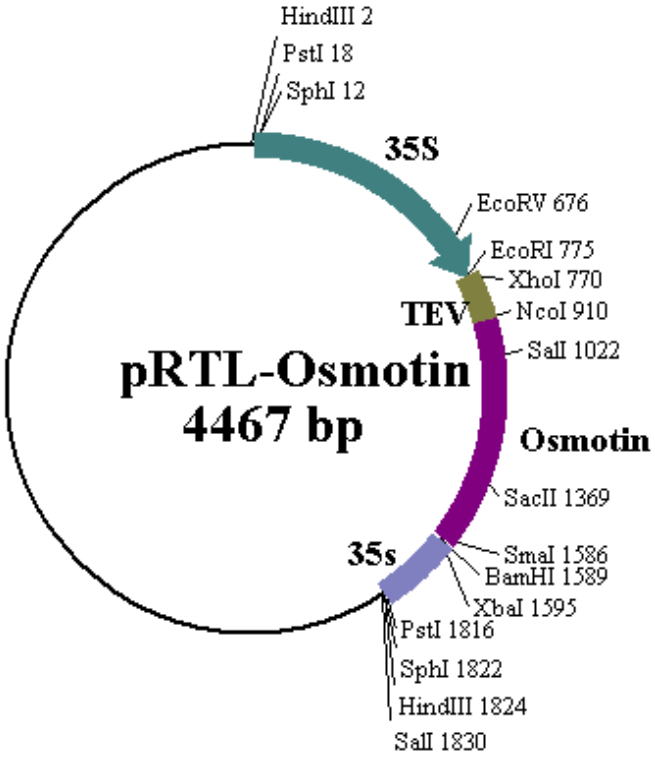


Figure 2: pRTL – Osmotin (Restrepo et al. 1990). Truncated tobacco osmotin gene under the control of the CaMV 35S promoter and terminator ligated into the binary vector pRTL2.

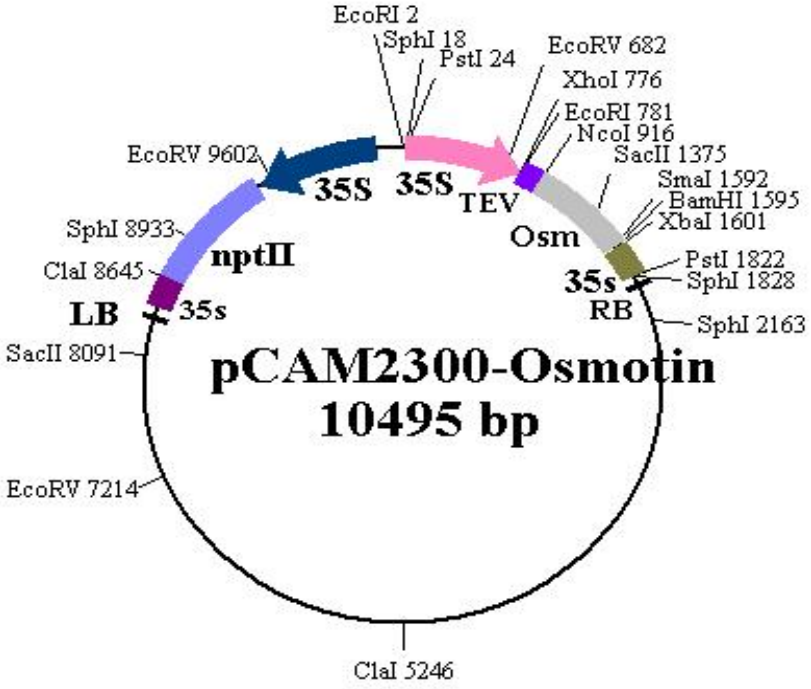


Figure 3: Plant transformation vector pCAMBIA2300-Osmotin (Parkhi et al. 2009). The expression cassette contains the truncated tobacco osmotin gene under the control of the CaMV 35S promoter and terminator, the translational enhancer from tobacco etch virus, and the Neomycin phosphotransferase (*nptII*) gene as a selectable marker.

Polymerase Chain Reaction (PCR)

Carrot genomic DNA was extracted following the protocol established by Chaudhry et al. (1999). The following thermal cycler and chemical reaction conditions were used for all PCR reactions using a Gene Amp® model 9700 Thermal Cycler. Each 25 µL reaction contained 2.5 µL 10X PCR buffer, 0.75 µL of 50 mM $MgCl_2$, 1 µL of 10 mM dNTP mix, 0.2 µL *Taq* polymerase, 1 µL of 10 mM from each primer, 1 µL DNA template, 17.55 µL ddH_2O. The reaction was heated to 95°C for 5 min and exposed to 34 cycles of 94°C for 30 s, 56°C for 45 s, and 72°C for 1 min and followed by 72°C for 10 min at the last cycle and held at 4°C. The reaction products were separated on 1% (w/v) agarose (Invitrogen, Carlsbad, CA) gel in 0.5X TBE, stained with ethidium bromide, and photographed. Duplicate RT-PCR was also used to confirm osmotin gene expression. Total RNA was extracted from carrot leaves using Spectrum™ Plant Total RNA Kit (Sigma-Aldrich). After treatment with DNase to remove DNA contamination, the RT reaction was carried out and cDNA was synthesized using the TaqMan® Reverse Transcription Reagents (Applied Biosystems, Branchburg, NJ) with oligo (dT) primers using the protocol provided by the supplier. The synthesized cDNA was utilized for the PCR with two sets of primers; the osmotin primer pair: Osm1-F: 5'-GCCGCCATGGGCAACTTGAGATCTTCT-3'; and Osm1-R: 5'-ACCCCCGGGCTAAGGACAAAAGATAACCCT-3', and carrot H1 histone primer pair: DcHis1-F: 5'-CTTGGCTGAATACGCTTTGGAC-3'; and DcHis1-R: 5'-

CCCGGGCTAAGGACAAAAGATAACCCT-3' and similar PCR thermal cycler settings as mentioned above.

Southern analysis to confirm genetic integration

The presence of transgene in the transformants was confirmed using Southern blot analysis. Genomic DNA was isolated from leaf tissue of transgenic and control plants' leaves using the protocol described by Chaudhry et al. (1999). The isolated genomic DNA (~12 µg) was digested with EcoRI and fractionated by electrophoresis on a 1% agarose gel in 0.5X TBE buffer at 22 V overnight and blotted on a Hybond-N^+ nylon membrane (GE Healthcare, Buckinghamshire, UK). For probe preparation, the osmotin gene in the plasmid pCAMBIA2300 was apmplified by PCR reaction and PCR products were separated on a 1% agarose gel by electrophoresis. The osmotin DNA fragment was purified from the agarose gel using the QIA quick Gel Extraction Kit (Qiagen Sciences, Germantown, MD). The blot was probed with radioactively labeled (α-P^{32} dCTP) osmotin using the Random Primer Labeling Kit (Invitrogen, Carlsbad, CA). The labeled blot was used to expose X-ray film at -80°C for 1-3 d to obtain an autoradiogram.

Western blot analysis for the expression of tobacco osmotin gene

Western blot was performed according to the protocol established by Sunilkumar et al. (2009) with some modifications. Total soluble protein (TSP) was extracted from carrot leaves following Song and Ahn (2010) and quantified using the Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA). Twenty five micrograms of TSP from transgenic and control plants were separated on 12.5% polyacrylamide SDS gel and blotted on a PVDF nylon membrane (Millipore Corporation, Bedford, MA). The membrane was blocked using blocking solution [(5% nonfat milk dissolved in 1X TBST (20 mM Tris (Sigma-Aldrich), 137 mM NaCl, pH (7.6), and 0.01% Tween-20)] for 1 h with continuous shaking. Antibodies raised against tobacco osmotin protein in chicken were used as the primary antibodies (1:500) which were purified from chicken eggs using Eggcellent^{TM} Chicken IgY Purification Kit (Pierce Biotechnology, Rockford, IL). The blot was treated with primary antibodies (anti-osmotin) overnight at 4°C with continuous shaking. Goat anti-chicken IgY HPR (Santa Cruz Biotechnology, Santa Cruz, CA) was used as the secondary antibody (1:5000). Antibody binding was detected using ECS chemiluminescence [(0.1 M Tris buffer (pH 8.9), 1.25 mM luminol (Sigma-Aldrich), 200 µM p-Coumaric (Sigma-Aldrich), 1 µL 30% H_2O_2)].

Short term drought treatment

Carrot seeds from three lines (LCT61-90, LCT61-100, and LCT61-147) along with the wild-type were sown in 280 well seedling trays at an average of 100 seeds per line. After 7 d, 36 seedlings from each line that showed as much uniform growth as possible were selected and transferred to 8.9 cm plastic pots to hold exactly 90 g of Sunshine #5 soil (SunGro Horticulture). Transgenic and control plants were grown in the growth chamber under previously mentioned conditions for 5 weeks. Because of the chance of having null-segregants as the transgenic lines were from the T1 generation, a duplicate PCR reaction was carried out using the osmotin gene and H1 histone primers. Only the plants that tested positive for the osmotin gene were selected and used for further analyses. Twenty plants each of transgenic line and control were selected for the short-term drought treatment experiment. Before the starting date of the experiment, all the plants participating in the experiment were watered to complete saturation. In the following days, water was withheld from half of the plants, while the other half was watered regularly. Plants were monitored daily for wilting. After the drought treatment was terminated, the plants were re-watered for recovery.

Determine relative water content

Relative water content (RWC) was determined following the protocol described by Schonfeld et al. (1988). The first fully expanded leaves from both watered and water-

stressed plants were collected to measure their fresh weight (FW). Then, each leaf was soaked in water for 24 h in sealed containers at room temperature. Excess water was removed by blotting the leaves on a filter paper and then reweighed to obtain turgid weights (TW). Further, the leaves were dried in an oven at 60°C for 72 h to determine the dry weights (DW). RWC was calculated using the formula described by Schonfeld et al. (1988):

$$RWC\ (\%) = [(FW - DW) / (TW - DW)] \times 100$$

Estimation of leaf H_2O_2 content

This experiment was performed following the protocol described by Kariola et al. (2005) using 3,3-diaminobenzidine (DAB) staining in detached carrot leaves with some modifications. Briefly, fully expanded leaves from both watered and water-stressed plants were collected and kept in 0.1% DAB (Sigma-Aldrich), solubilized in 10 mM MES buffer (pH 6.5), overnight. Chlorophyll was completely removed with 100% ethanol at 65°C for 3 h and the leaves were embedded in 70% ethanol for observation using the M^2Bio Fluorescence Microscope (Kramer Scientific Corporation, Amesbury, MA). The intensity of spots resulting from ROS accumulation was quantified using the WinRHIZO Pro 2007 software (Regent Instruments Inc., Canada).

Estimation of malondialdehyde (MDA) content in leaf tissue

A 150 mg (midrib excluded) of fully expanded leaf sample was homogenized in 5 mL of 10% trichloroacetic acid (TCA) (Sigma-Aldrich) and the extract was centrifuged at 10,000 rpm for 10 min. The reaction was carried out by adding 2 mL supernatant to 3 mL of 0.6% thiobarbaturic acid (Sigma-Aldrich) dissolved in 10% TCA and incubated at room temperature for 2 h. After boiling at 100°C for 1 h and cooling down to room temperature, the OD was measured spectrophotometrically at 450 nm, 532 nm, and 600 nm (Quan et al. 2004). The malondialdehyde (MDA) content was estimated using the following formula:

$$C\ (\mu mol / L) = [6.45(OD_{532} - OD_{600})] - 0.56\ OD_{450}$$

Measurement of ion leakage in leaf tissue

A modified method of Fan et al. (1997) was followed to measure the ion leakage following a short-term drought treatment. A 200 mg-size, fully expanded leaf tissue was collected from each watered and water-stressed carrot plant in a 50 mL centrifuge tube containing 15 mL of 0.4 M mannitol. The tubes were incubated (with gentle shaking) at room temperature for 3 h and the conductivity of the bathing solution was measured using an Oakton CON 11 Series conductivity meter (Eutech instruments, Malaysia). After this reading, the samples were boiled for 10 min and the total conductivity of the

bathing solution was determined. Membrane ion leakage was expressed in terms of initial conductivity of the bathing solution as a percentage of the total conductivity.

Estimation of proline content in leaf tissue

Proline levels in tissues were determined according to Bates (1973) and Ringel et al. (2003). One hundred milligrams of fully expanded leaf sample from each watered and water-stressed carrot plant was collected in a 2 mL microfuge tube containing 1.7 mL of 3% sulfosalicylic acid (Sigma-Aldrich). Samples were homogenized with the aid of stainless steel beads and centrifuged. A 1 mL aliquot of supernatant was mixed with 1 mL of acid ninhydrin (Sigma-Aldrich) and 1 mL of glacial acetic acid in a 20 mL size tube and incubated at 100°C for 1 h. Consequently, the reaction was terminated by incubating the tubes on ice. An aliquot of 2 mL of toluene was added to each tube and the mixture was vortexed for 10 s. One milliliter of the upper, toluene phase containing the chromophore was collected and read at 520 nm in a quartz cuvette specrophotometrically. Tissue proline concentrations were estimated based on a standard curve (0–100 µg/mL) for proline and are presented as µg proline/g FW according to the following equation:

$$\mu g \text{ proline/g FW} = [(\mu g \text{ proline/mL} \times 3.7)/100 \mu g \text{ tissue}] \times 10$$

3. RESULTS AND DISCUSSION

Generation of transgenic plants

We have transformed carrot plants with tobacco osmotin gene lacking a sequence encoding 20 amino-acids at the C-terminal end with the aim of producing superior transgenic lines in term of drought tolerance. This truncated version of the gene has been reported to confer tolerance to salinity, drought, and certain fungal species (Singh et al., 1987; LaRosa et al., 1989; Abad et al. 1996). However, a recent study on cotton plants did not find enough evidence to support its antifungal or salinity-tolerance properties (Parkhi et al., 2009), however, the truncated osmotin did confer drought tolerance to the transformants. Using the *Agrobacterium*-mediated transformation method, we produced 123 putative transgenic plants from 90 different transformed callus lines. For the initial screening of the putative carrot transformants, PCR analysis was performed to detect the presence of tobacco osmotin gene in their genomic DNA. The transgenic lines were identified by the presence of an amplicon of 0.36 Kb size. Fig.4 shows results from one such PCR analysis.

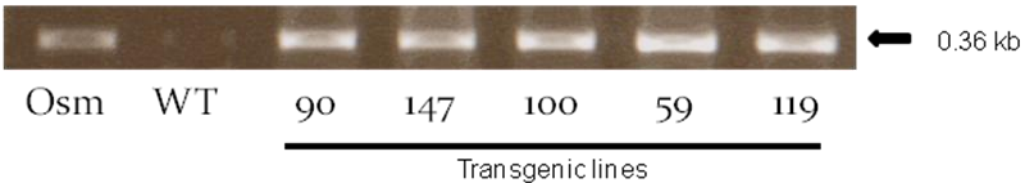


Figure 4: PCR analysis for T0 transgenic plants using osmotin gene-specific primers. Positive control (Osm), wild-type (WT), Transgenic lines (LCT61- 90,147,100, 59,119). Only the transgenic lines showed the amplified fragment corresponding to the coding region of the osmotin transgene.

It should be noted that PCR analysis sometimes can provide false positive results due to *Agrobacterium* contamination of the regenerated plants. To prevent *Agrobacterium* growth, transformed calli are usually kept for relatively long time on media that contain appropriate antibiotics. In our case, we kept our calli on MS medium containing 100 mg/L kanamycin and 500 mg/L cefotaxime for about two months. To examine whether our transgenic events were harboring *Agrobacterium* within their tissues, two different assays were performed. The first assay was performed by taking a leaf from each the transgenic event, cutting it into smaller pieces, and placing them on yeast extract peptone (YEP) medium [10 g/L yeast extract (Difco Laboratories Inc.), 10 g/L Bacto peptone (Difco Laboratories Inc.), 5 g/L NaCl, pH 7.0] at 28°C. After 3 d, *Agrobacterium* growth was observed around some of the leaf pieces suggesting that PCR positive results may have been obtained due to survival of *Agrobacterium* within the tissues (data not shown). The second assay was performed by conducting a PCR reaction using a pair of primers (pC2300-F: 5'- GAGCTGCCCATTCTTGAGTC-3' and pC2300-R: 5'-ACCGCGTACATCTTCAGCTT-3') specific to the pCAMBIA2300 vector's backbone. At least one plants showed positive PCR band suggesting the presence of *Agrobacterium*

(Fig.5). Therefore, the results from both assays suggested that the time period of 2 months for calli maintenance on appropriate antibiotics might not be enough to eliminate all the *Agrobacteria*, especially those that live deep within the callus clusters. The results also show that PCR is not always a reliable tool to investigate the presence of trasgenes in target genomes especially when using the *Agrobacterium*-mediated transformation method.

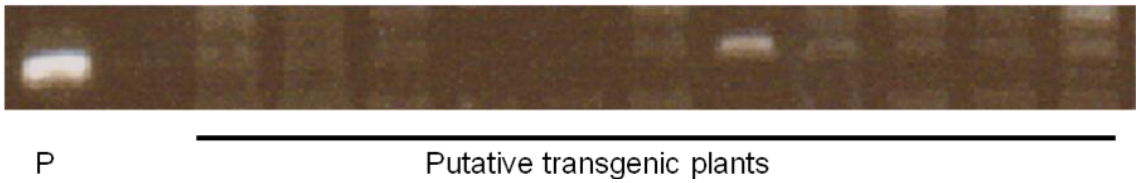


Figure 5: PCR analysis to detect *Agrobacterium* contamination in the putative transgenic plants using pCAMBIA2300 backbone specific primers. One plant showed a positive amplification indicating the presence of *Agrobacterium* contamination. Positive control from the *Agrobacterium* plasmid (P).

Therefore, we used Southern blot analysis to completely verify the presence and integration of the tobacco osmotin gene in the *D. carota* genome. Genomic DNA from the transgenic and wild-type plants was digested with EcoRI and the blots were hybridized with an osmotin gene-specific probe. The analysis revealed hybridization bands only for the transgenic lines and not in the lane containing in the wild-type DNA (Fig.6).

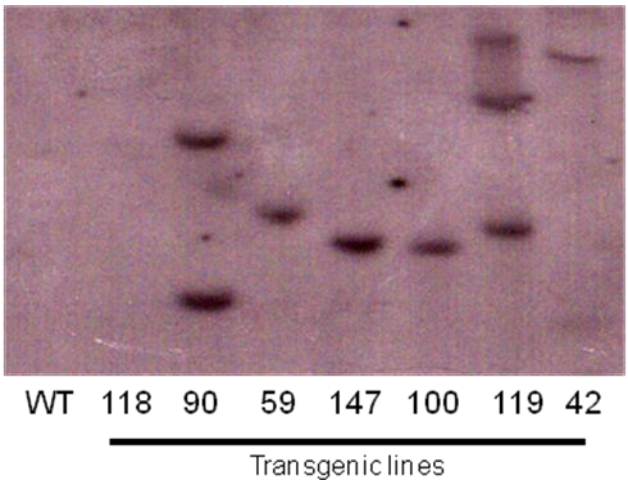


Figure 6: Southern blot analysis of putative transgenic plants. Genomic DNA was digested with EcoRI and the blot was probed with radio-labeled osmotin gene-specific probe. Negative control from untransformed carrot wild-type plants (WT).

Southern blot analysis revealed that, out of 123 putative transgenic plants produced following transformations, only 44 plants tested positive for tobacco osmotin gene integration. Similar transformation efficiency was observed by Pawlicki et al. (1992) with carrot variety Chantenay when transforming with *Agrobacterium tumefaciens*. These 44 plants were subjected to a 2-month long cold treatment at 4°C to induce flowering. Only 13 plants flowered following the vernalization treatment. In order to analyze the osmotin gene expression, duplicate RT-PCR analysis was performed. Out of 13 plants that showed the formation of flower stalks, only 5 transgenic lines expressed the osmotin transgene (Fig.7).

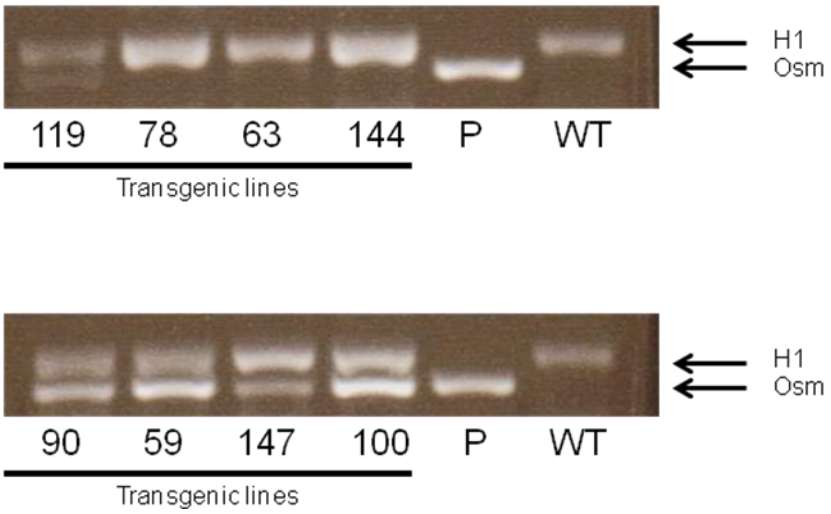


Figure 7: Duplicate RT-PCR analysis on T0 transgenic lines using osmotin gene-specific primers along with carrot H1 histone gene-specific primers. Positive control from osmotin plasmid (P), negative control from untransformed carrot wild-type plants (WT). Histone (H1) was used as an internal control for the synthesized cDNA. Osmotin gene corresponding band (Osm).

In summary, approximately one year and 4 months was needed to produce T1 seed (Fig.8). Peterson and Simon (1986) reported that wild carrot (Queen Anne's Lace) usually completes its seed-to-seed life cycle in a year under field condition. Considering the tissue culture period, the time-length we experienced in producing T1 seed was reasonable.

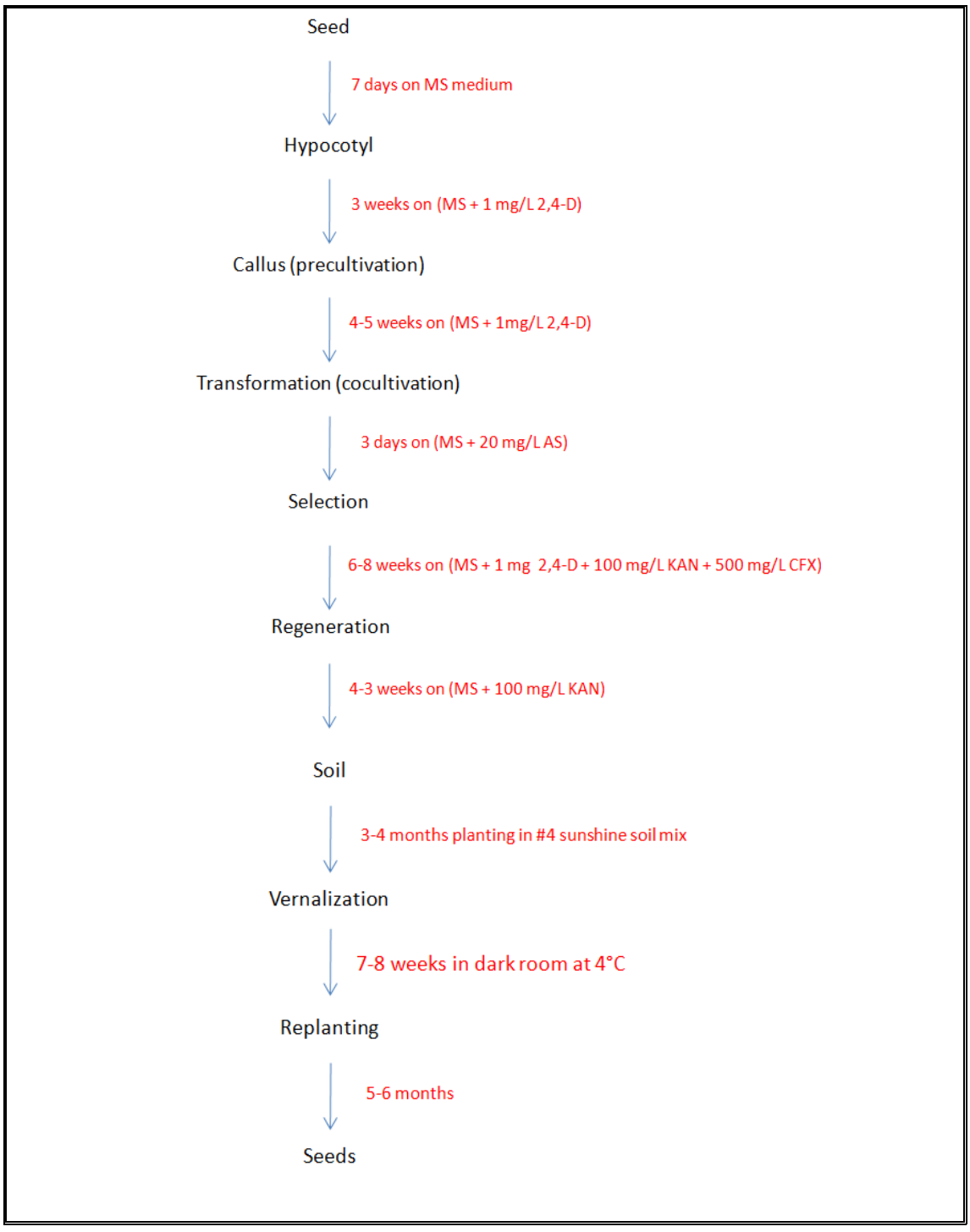


Figure 8: A schematic representation of the stages from seed to seed through tissue culture for the production of T1 generation. Acetosyringone (AS), kanamycin (KAN), cefotaxime (CFX).

Screening for osmotin in the T1 transgenic plants

As previously mentioned, only 5 out of 13 transgenic plants that were able to flower showed the expression of the osmotin gene and were able to produce seeds. Carrot is a cross-pollinated crop that relies mostly on insects, although self pollination can also occur (Rubatzky et al., 1999). In our case, self-pollination was the only option to maintain the purity of transgenic lines. Simon (1993) reported that selfing can lead to a reduced quality and quantity of carrot seeds. From 5 transgenic lines that showed osmotin gene expression, only 3 transgenic lines (LCT61-90, LCT61-100, and LCT61-147) gave us sufficient amount of seeds to conduct the physiological and biochemical analyses.

The presence of osmotin gene and its inheritance in the T1 transgenic lines was confirmed by Southern blot analysis. Genomic DNA was digested with EcoRI and the blot was hybridized using the osmotin gene-specific probe (Fig.9). All the T1 transgenic lines were positive for the presence of the transgene while no hybridization was detected in the wild-type plants. The banding pattern similarity between transgenic lines LCT61-100 and LCT61-147 does not necessarily mean that they are descendent from the same independent line. Normally, when *Agrobacterium* strains infect the host, their T-DNA will get integrated at random positions within the target genome (Chilton et al., 1977). During restriction digestion, and depending on the restriction enzymes used, these different positions may provide fragments of similar sizes in rare cases. Lines LCT61-100 and LCT61-147 appear to be such a case.

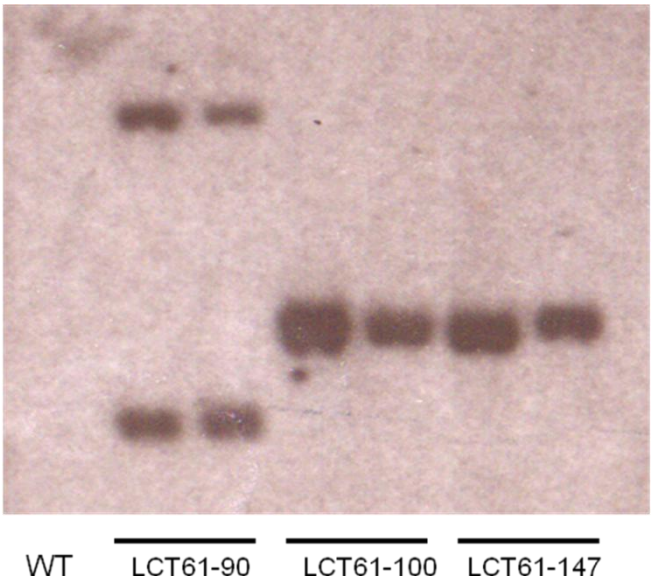


Figure 9: Southern blot analysis of genomic DNA from leaves of T1 transgenic plants. The DNA was digested with EcoRI and the blot was probed with osmotin gene-specific probe. Negative control from untransformed carrot wild-type plants (WT).

The expression of the osmotin gene was examined by Western blot analysis on leaf tissue extracts from selected transgenic lines. Total soluble protein was extracted and an equal amount of 25 µg per sample was loaded in each well. The results showed clearly the expression of osmotin protein in carrot transgenic lines. No reaction was detected between the wild-type protein extract and the anti-osmotin antibody (Fig.10).

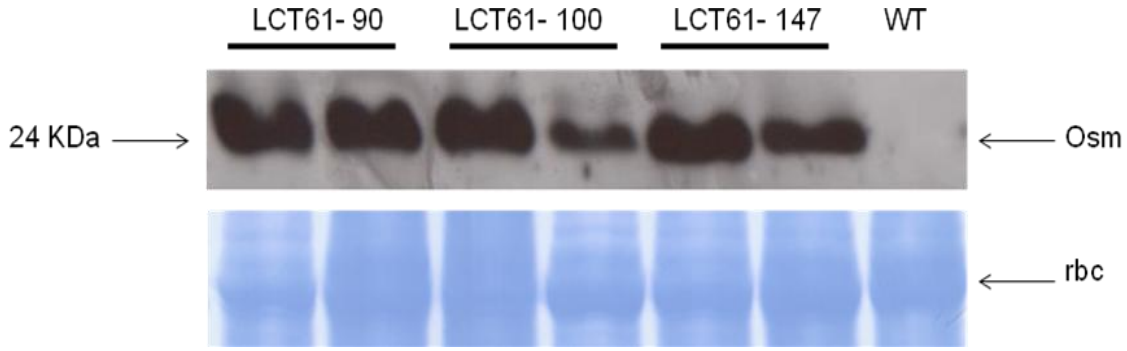


Figure 10: Western blot analysis for tobacco osmotin protein in T1 plants obtained from three different transgenic lines. Wild-type (WT), osmotin corresponding band (Osm), rubisco (rbc). Approximately twenty five microgram of total protein was loaded in each lane. Coomassie blue stained gel is shown as loading control.

Segregation analysis for T1 transgenic plants

Plants from each transgenic line were used to examine their tolerance to experimental drought conditions. T1 plants were initially tested by placing them on half strength MS medium containing 100 mg/L kanamycin. Only the plants containing the LCT61 cassette with *nptII* selectable marker, that confers resistance to kanamycin, were able to survive and perform normally, while the wild-type seeds and null-segregants failed to germinate (Fig.11a). Further screening for the presence of osmotin transgene was done by duplicate PCR using the osmotin and H1 histone specific primers. The plants that did not show the presence of osmotin were eliminated from the experiment (Fig.11b).

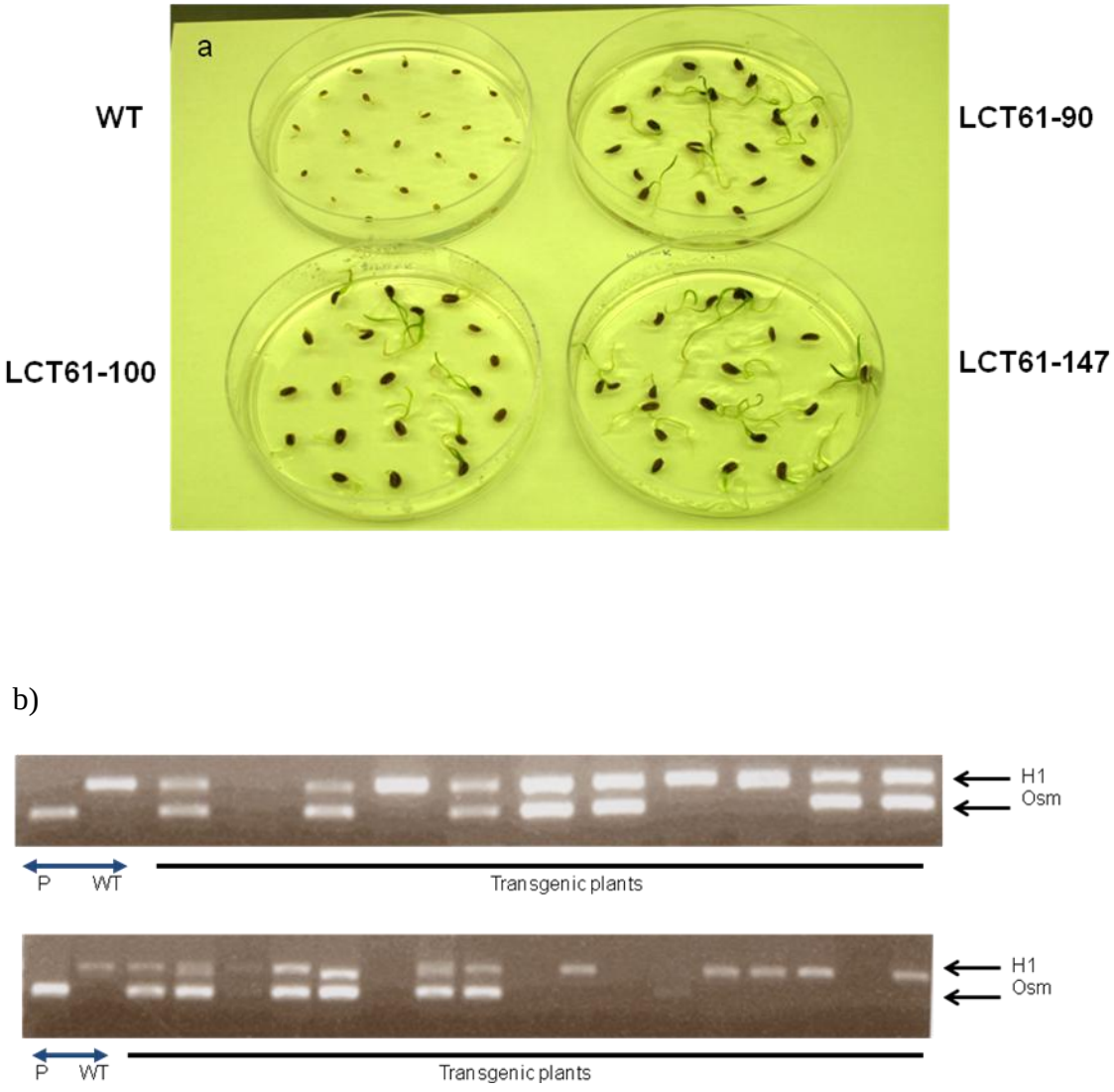


Figure 11: Selection of transgenic plants prior to the short-term drought experiment. a) T1 transgenic and control seeds germinated on MS medium containing 100 mg/L kanamycin. Only plants with the transgene cassette insertion were able to survive while all the wild-type and null segregant seeds failed to germinate. b) Duplicate PCR screening using the osmotin and H1 histone gene-specific primers. Positive control (P), wild-type (WT), osmotin corresponding band (Osm), *D. carota* H1 Histone (H1).

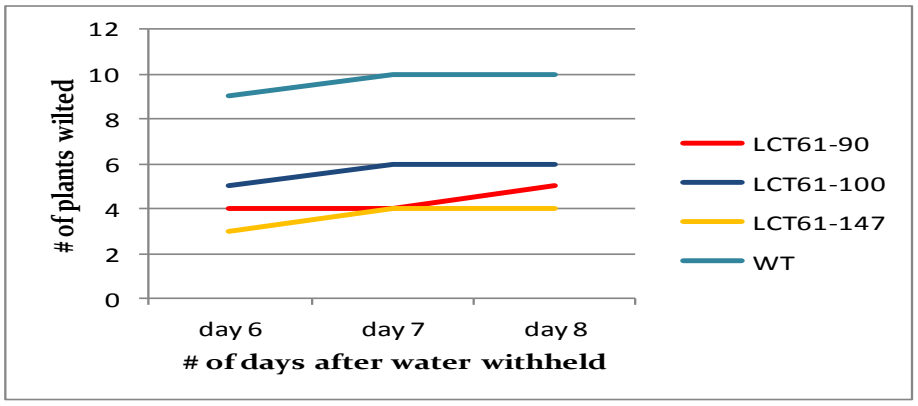
Evaluation of transgenic plants subjected to drought stress

To evaluate the response to drought stress under growth chamber condition, three transgenic lines and the wild-type plants were subjected to drought conditions by withholding watering for 6 d. On day 6, nine out of ten plants from the wild-type showed severe wilting symptoms, while less wilting symptoms were observed in the transgenic line (Fig.12a). By day 7, all the wild-type plants had wilted while the wilting status of the transgenic lines was the same of that observed on day 6 (Fig.12b).

a)

Figure 12: a) Wilting status of wild-type (WT) and transgenic carrot plants after 6 d of water stress. Transgenic plants showed less severe wilting when compared to the wild-type. b) Line graph showing wilting in WT plants and transgenic carrot plants subjected to water stress for 6 d. c) Response to re-watering after 6 d of drought stress. Top panel shows stressed plants after 6 d of withholding water and bottom panel shows one day of re-watering after drought stress.

b)



c)

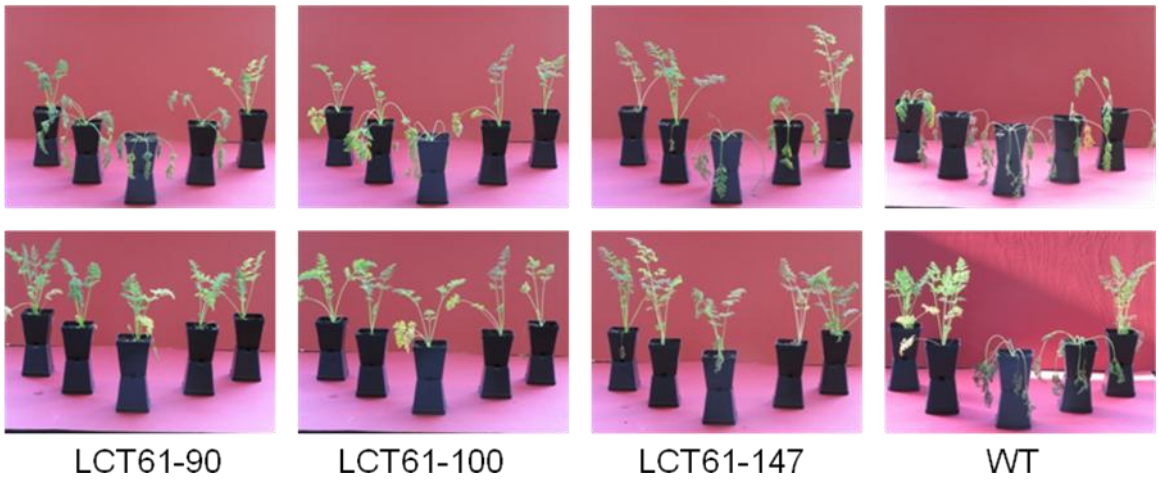


Figure 12: continued

We also examined the effects of re-watering after a drought stress treatment was applied in a different set of experiment. Five plants from each transgenic line along with the wild-type plants were subjected to water deficit conditions for 6 d. After the wilting signs became visible, the plants were re-watered and monitored for recovery. All the

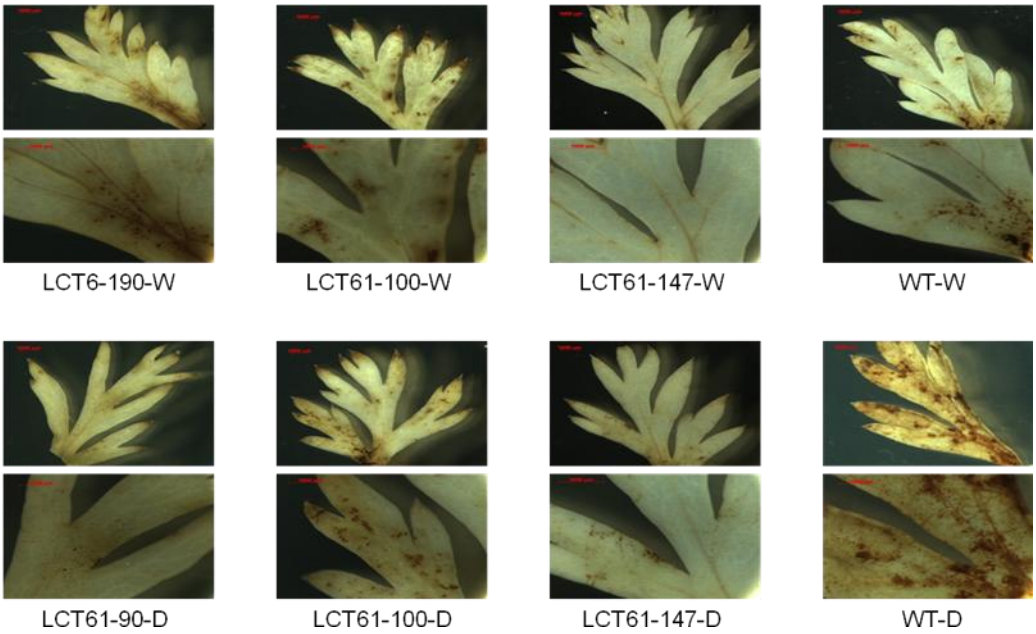
transgenic lines showed fast and full recovery while two of the wild-type plants failed to recover and died due to drought stress (Fig.12c). Lower and slower rates of leaf wilting are a common feature for many drought-tolerant cultivars (Oya et al., 2004; Abraham et al., 2004; Ober et al., 2005). Similar results were also obtained during the induction of 'tolerant' proteins induced by drought conditions, such as dehydrins (Stupnikova et al., 2002), aquaporins (Borstlap, 2002), dcTLP (Jung et al. 2005). In addition to the regulatory involvement of these proteins in controlling the water movement, they also play a role in accumulating compatible solutes and osmolytes such as sucrose, polyols (mannitol, sorbitol, pinitol), proline, trehalose, fructans, and betaines which control the stomatal conductance and maintain cells turgor pressure (Smirnoff, 1998).

Hydrogen peroxide (H_2O_2) accumulation in plants under drought stress

Reactive oxygen species (ROS) including hydrogen peroxide (H_2O_2), superoxide anion, singlet oxygen ($^1O_2$), and hydroxyl radicals are believed to play an important role in plant inter- and intracellular signaling (Doke, 1997; Foyer and Noctor, 1999), and are considered to be a necessary component for the correct functioning of plants metabolic processes (Halliwell and Gutteridge, 1989; Asada, 1999). However, a high level of these products due to different stresses can damage cell structures and restrain cell function (Halliwell and Gutteridge, 1989; Elstner, 1991; Asada, 1999). Therefore, the accumulation of such products is a good indicator of the plant's health under stress conditions. To investigate the level of H_2O_2 accumulation, leaves from the wild-type and

plants from each of the transgenic lines, subjected to drought stress by withholding water for 6 d, were stained with DAB and examined visually. Large areas of dark-brown precipitation were seen on the wild-type plants under water stress conditions, while smaller percentage of such brown spots was observed on the transgenic lines (Fig.13a). Automated colorimetric analysis revealed that the wild-type plants under water stress conditions showed browning in 46% of their total leaf area, while only 4, 5, and 11% of browning were observed in lines LCT61-147, LCT61-90, and LCT61-100, respectively (Fig.13b). An et al. (2011) found that a temperature-sensitive mutant of *Capsicum chinense*, *sy-2*, showed high accumulation of H_2O_2 when subjected to a prolonged period of relatively low temperature. Moreover, a zebra-necrosis (zn) mutant of rice exhibited dark-brown spots in the yellow sectors of their leaves during photoinhibition due to increased levels of H_2O_2 (Li et al., 2010). In plants, ROS is constantly produced in the peroxisomes, mitochondria, and chloroplasts as by-products of aerobic reactions (Ahmad et al., 2008; Apel and Hirt, 2004). Therefore, by exceeding their scavenging limits, they start to act as cytotoxins attacking the surrounding cells and causing such necrotic lesions.

a)



b)

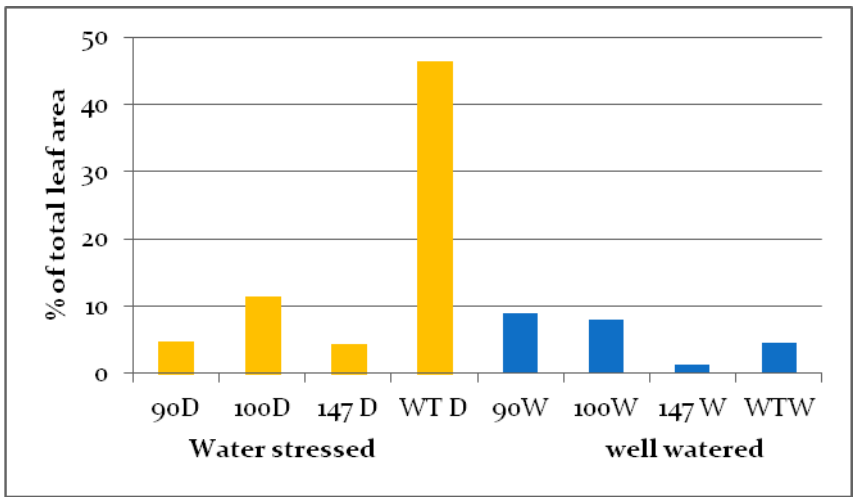


Figure 13: Accumulation of Hydrogen peroxide (H_2O_2) in plants subjected to drought stress. a) Leaf section images from wild-type (WT) and transgenic lines after 6 d under normal conditions (top panel) and water stress conditions (bottom panel) showing H_2O_2 accumulation with 3,3-diaminobenzidine (DAB) staining. b) Brown area percentage relative to the total leaf area due to H_2O_2 accumulation after 6 d of water stress.

Estimation of malondialdehyde (MDA) in plant leaves under drought stress

MDA is generated as secondary breakdown products of lipid peroxidation caused by high levels of reactive oxygen species (ROS) during oxidative stresses (Davey et al., 2005, Shulaev and Oliver, 2006, Zlatev et al., 2006). Increased levels of MDA and other relative aldehydes can alter DNA, RNA, and proteins structure due to Schiff's base addition reaction, although it can also act as a secondary messenger during abiotic stress which up-regulate response genes (Esterbauer et al., 1991; Vollenweider et al., 2000; Farmer et al., 2003; Weber et al., 2004). MDA is widely used as a marker for lipid oxidation damage caused by various types of biotic and abiotic stresses (Esterbauer and Cheeseman, 1990; Sairam et al., 1998; Kenton et al., 1999; Scarpari et al. 2005).

The levels of MDA in transgenic and control carrot plants under water stress conditions were estimated and the results are presented in Fig.14. All three transgenic lines showed lower level (P <0.01) of MDA accumulation comparing to the controls. However, no significant difference between the control and transgenic lines was observed under normal watering conditions. Similar observations were reported in an investigation on field-grown sage plants under drought stress conditions, suggesting a relationship between drought and oxidative stress (Munne-Bosch et al. 2001).

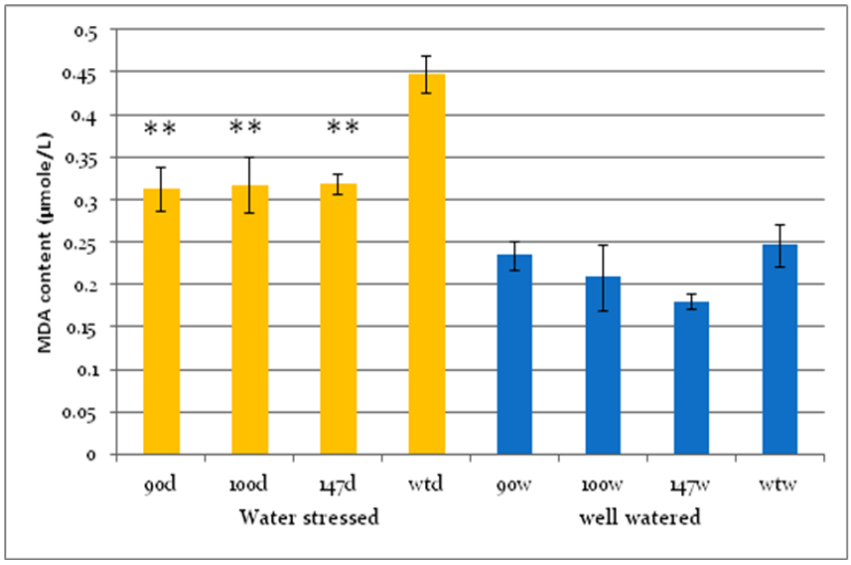


Figure 14: Malondialdehyde (MDA) levels in wild-type (WT) and transgenic carrot plants subjected to water stress conditions (yellow bars) and normal condition (blue bars) for 6 d. The data represent mean (n=5) ± SE. (**P<0.01). In each type of treatment, WT was compared to the transgenic lines treated in the same manner.

Relative water content of carrot leaves under drought stress

Leaves from the wild-type and three transgenic lines subjected to 6 d of water stress were examined for their ability to conserve water. Carrot transgenic lines LCT61-147, LCT61-90, and LCT61-100 showed significantly higher water content in their leaves ($P < 0.01$, $P < 0.01$, and $P < 0.05$, respectively) when compared with the wild-type counterparts. Their relative water contents were 20, 18, and 11% higher than the wild-type plants in lines LCT61-147, LCT61-90, and LCT61-100, respectively (Fig.15).

No significant difference was observed between transgenic lines and controls when the plants were well-watered.

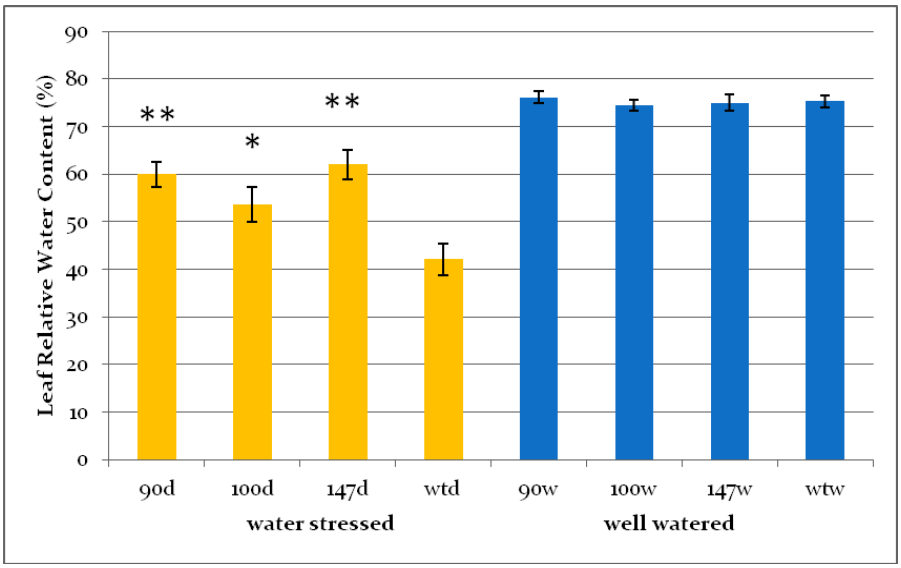


Figure 15: Relative water content in wild-type (WT) and transgenic carrot plants subjected to water stress conditions (yellow bars) and normal conditions (blue bars) for 6 d. The data represent mean (n=5) ± SE. (*P<0.05, **P<0.01). In each type of treatment, WT was compared to the transgenic lines treated in the same manner.

Sinclair and Ludlow (1985) proposed that RWC can be considered a better indicator than water potential when it comes to revealing the health status of a plant cell. This is reasonable because water is a major component necessary for almost every inter-intracellular biochemical reaction. Different mechanisms were hypothesized to be involved in higher degree of drought tolerance including cell membrane stability, osmotic adjustment, maintenance of redoxin homeostasis and detoxification, and protection of important macromolecules from degradation (Hu et al., 2006). Osmotin's suggested mode of action is either by facilitating the compartmentalization of solutes or

by altering the plant structure and metabolism which then leads to the osmotic adjustment of the plant cell (Singh et al., 1987).

Measurement of leaked ions in the leaves of plants under drought stress

The ability of cell membranes to maintain their stability and integrity during stresses is considered a major characteristic of drought tolerance (Bajji et al., 2001). Lipid peroxidation caused by high levels of ROS during a stress can damage the cell membranes and lead to electrolyte leakage (Sreenivasulu et al., 2000; Lauriano et al., 2000; Apel and Hirt, 2004; Khanna-Chopra and Selote, 2007). Therefore, the degree of cell membrane injury caused by drought stress can be measured by quantifying ion leakages from the plant tissue. In order to do that, a conductivity meter was used to measure the conductivity of the aqueous medium bathing the leaves of plants that were subjected to 6 d of water withholding. The results revealed that even under unstressed conditions, transgenic plants showed less ion leakage ($P < 0.05$) than the wild-type plants. Furthermore, significantly higher ($P < 0.01$) ion leakage was observed in the wild-type plants subjected to water stress when compared to the transgenic lines (Fig.16). The relative conductivity values obtained from the three transgenic lines were 10.8, 13.2, and 13.7% for lines LCT61-90, LCT61-100, and LCT61-147, respectively which were significantly different ($P < 0.01$) from the wild-type value of 29.4%. These results suggest that transgenic plants suffered less damage to their cellular membranes under drought stress. Many studies have used ion leakage to assess the severity of

damage to the plant cells when encountering unfavorable conditions. del Pozo and Lam (1998) used electrolyte leakage assay to compare the relative amount of cell death in plant tissues when studying the programmed cell death response of plants towards pathogens. Drought-tolerant cultivars of bean (Zlatev et al., 2006), wheat (Sairam et al., 1998) and maize (Pastori and Trippi, 1992) showed high cell membrane stability and low levels of reactive oxygen species. Our results clearly indicate lower levels of ROS and ion leakage in the transgenic plants when subjected to dehydration conditions, and thus support our conclusion of higher membrane stability in the transgenic plants.

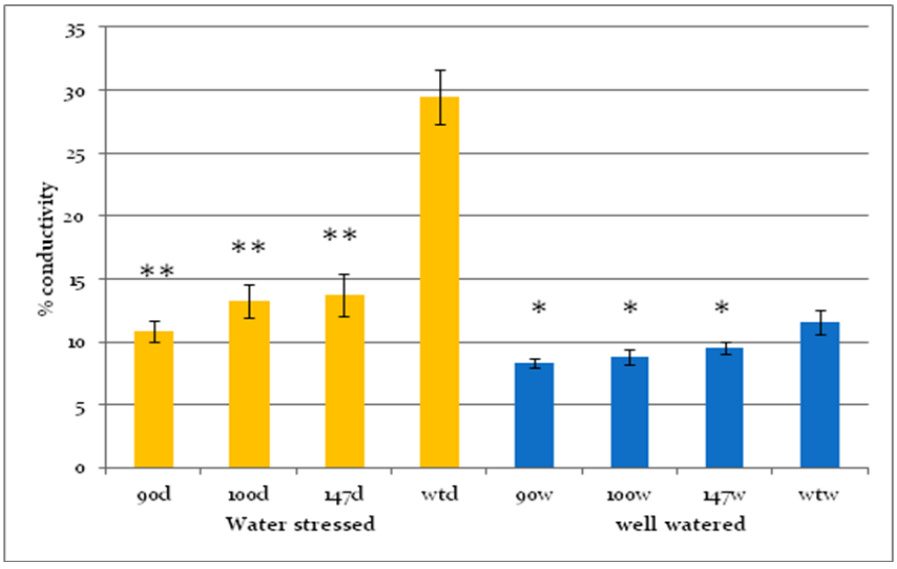


Figure 16: Measurement of Ion leakage in wild-type (WT) and transgenic carrot plants subjected to water stress conditions (yellow bars) and normal condition (blue bars) for 6 d. The data represent mean (n=5) ± SE. (*P<0.05, **P<0.01). In each type of treatment, WT was compared to the transgenic lines treated in the same manner.

Estimation of proline content in the leaves of plants under drought stress

As a part of their defense system, plants have the ability to accumulate specific metabolites in response to different stresses. One of these metabolites is the amino acid, proline. This compound has been reported to accumulate to a level even more than 100 times the normal concentration in a large variety of plants under stress conditions (Barnett and Naylor, 1966; Thomas et al., 1992; Wanner and Junttila, 1999). High accumulation of proline is believed to enhance drought tolerance (Stewart and Larhar, 1980; Dib et al., 1994; Yamada et al., 2005) and therefore considered a good indicator of drought tolerance. Tobacco plants expressing the osmotin gene were shown to produce high level of proline whether under stressed or normal condition (Barthakur et al. 2001). There are two possible ways for the protective mode of proline, firstly, by protecting the enzymes structure from denaturation due to heat, cold, and salinity (Rajendrakumar et al., 1994; Pollard and Wyn Jones, 1979) and, secondly, by scavenging ROS accumulation during oxidative stresses (Smirnoff and Cumbes, 1989). Proline content was measured in the leaves of transgenic and control plants under well-watered and water stressed conditions. In well-watered condition, the level of proline was very low in all of the plants under investigation, with no significant difference between them. When water was withheld for 6 d, the level of proline increased in all the transgenic and control plants as shown in Fig.17. However, the expression of the osmotin transgene did not result in a significant increase in the proline levels of the transformants with respect to the controls, as has been reported by others. In fact, the wild-type proline level was

significantly higher (P <0.01) than lines LCT61-90 and LCT61-100 and slightly lower than line LCT61-147. The proline levels were 14.8, 19.4, 26.3, 23.9 µg/g fresh weight in LCT61-90, LCT61-100, LCT61-147, and the wild-type, respectively. Several studies demonstrated the beneficial effects of higher proline content of the plants in terms of their ability to withstand drought and salinity stress (Krishnamurthy, 1991; Kavi Kishor et al., 1995; Iyer and Caplan, 1998; Ramanjulu and Sudhakar, 2000; Parkhi et al., 2009; Goel et al., 2010). Thus our results on the degree of proline accumulation and the relative ability of the plant to tolerate water stress are not in line with earlier reports. A possible explanation for this result is that, as opposed to other species, carrot does not totally rely on high proline levels to protect itself from the drought. It is also possible that the studies showing a lack of correlation between proline accumulation and drought tolerance of a plant have not been published.

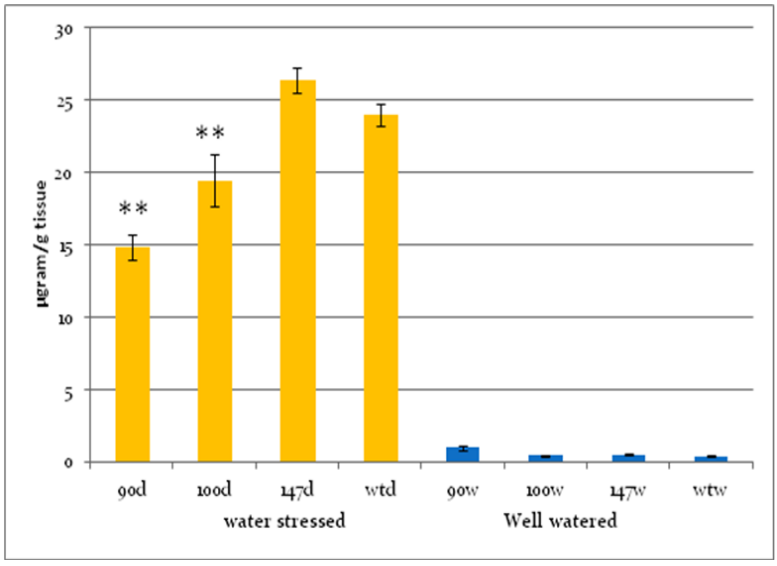


Figure 17: Proline levels in wild-type (WT) and transgenic carrot plants subjected to water stress conditions (yellow bars) and normal condition (blue bars) for 6 d. The data represent mean (n=5) ± SE. (**P<0.01). In each type of treatment, WT was compared to the transgenic lines treated in the same manner.

4. CONCLUSIONS

The tobacco osmotin gene identified and isolated by Singh et al. (1985) and reported to confer tolerance to drought, salinity, and certain pathogenic fungi (Singh et al. 1987; LaRosa et al. 1989; Abad et al. 1996) was successfully introduced into the carrot genome using the *Agrobacterium*-mediated transformation method (Rathore et al. 2006) as confirmed by Southern blot analysis. However, only 5 transgenic plants showed the expression osmotin mRNA and formed flowers. The secretion of osmotin protein was also confirmed by Western blot analysis that showed stable and abundant production of the protein. In response to a simulated drought stress and re-watering, several parameters were examined including wilting status, ROS accumulation, electrolytes leakage, relative water content, and free proline levels. In response to six days of withholding of watering, the transgenic lines showed lesser levels and slower wilting compared to the wild-type plants. In addition, transformants exhibited faster and full recovery from wilting upon the termination of drought conditions. ROS accumulation, including hydrogen peroxide and malondialdehyde levels, were significantly lower in the transgenic lines compared to the wild-type counterparts providing an evidence of less oxidative damage due to the dehydration treatment. The transgenic lines were able to conserve more water in their tissue when subjected to drought condition for 6 d. Moreover, the transgenic plants showed significantly less ion leakage compared to the wild-type plants. Taken together, these results suggest an elevated degree of drought tolerance in the transgenic lines of carrot expressing the tobacco osmotin gene. Rubatzky

et al. (1999) showed that carrot seedlings are sensitive to *Fusarium* spp. which can cause wilting, stem base tissue decay, and seedling death. They also found that *Phytophthora* spp. can cause severe damage to carrot roots and reduce their quality. Abad et al. (1996) reported that these two fungi species were highly sensitive to tobacco osmotin. Therefore, it would be useful to test these same transgenic lines for their ability to resist these two fungal pathogens. If found to be effective, it will significantly increase the yield and quality of carrot plants.

REFERENCES

Abad LR, D'Urzo MP, Liu D, Narasimhan ML, Reuveni M, Zhua JK, Niua X, Singh NK, Hasegawaa PM, Bressan RA (1996) Antifungal activity of tobacco osmotin has specificity and involves plasma membrane permeabilization. Plant Sci **118**:11–23

Abraham EM, Huang B, Bonos SA, William AM (2004) Evaluation of drought resistance for Texas bluegrass, Kentucky bluegrass, and their hybrids. Crop Sci **44**:1746–1753

Ahmad P, Sarwat M, Sharma S (2008) Reactive oxygen species, antioxidants and signaling in plants. J. Plant Biol **51**: 167–173

Amar L, Relnhold L (1973) Loss of membrane transport ability in leaf cells and release of protein as a result of osmotic shock. Plant Physiol **51**: 620- 625

An SJ, Pandeya D, Park SW, Li J, Kwon JK, Koeda S, Hosokawa M, Paek NC, Choi D, Kang BC (2011) Characterization and genetic analysis of a low-temperature-sensitive mutant, sy-2, in *Capsicum chinense*. Theor Appl Genet **122**:459–470

Angeli SD, Altamura MM (2007) Osmotin induces cold protection in olive trees by affecting programmed cell death and cytoskeleton organization. Planta **225**:1147–1163

Apel K, Hirt H (2004) Reactive oxygen species: metabolism, oxidative stress, and signal transduction. Plant Biol **55**:373–399

Asada K (1999) The water–water cycle in chloroplasts: scavenging of active oxygens and dissipation of excess photons. Plant Physiol **50**:601–639

Bajji M, Kient JM, Lutts S (2001) The use of the electrolyte leakage method for assessing cell membrane stability as a water stress tolerance test in durum wheat. Plant Growth Regulation **00**:1-10

Barnetr T, AlsSchuler M, McDaniel CN, Mascarenhas JP (1980) Heat shock induced proteins in plant cells. Dev Genet **1**: 331-340

Barnett NM, Naylor AW (1966) Amino acid and protein metabolism in Bermuda grass during water stress. Plant Physiol **1**: 1222-1230

Barthakur S, Babu V, Bansal KC (2001) Overexpression of osmotin induces proline accumulation and confers tolerance to osmotic stress in transgenic tobacco. J Plant Biochem Biotechnol **10**:31–37

Bates LS (1973) Rapid determination of free proline for water-stress studies. Plant Soil **39**:205–207

Bol JF, Linthorst HJM, Cornelissen BJC (1990) Plant pathogenesis-related proteins induced by virus infection. Annu Rev Phytopathol **28**:113-138

Borstlap AC (2002) Early diversification of plant aquaporins. Trends Plant Sci **7**:529–530

Bryant EA (1991) Natural Hazards. In TE Downing, eds, International Journal of Climatology, Vol 13. Cambridge University Press, Cambridge, pp 344-346

Bryngelsson T, Green B (1989) Characterization of a pathogenesis-related, thaumatin-like protein isolated from barley challenged with an incompatible race of mildew. Physiol Mol Plant Pathol **35**: 45-52

Casas AM, Nelson DE, Raghothama KG, Paino DUrzo M, Singh NK, Bressan RA, Hasegawa PM (1992) Expression of osmotin-like genes in the halophyte *Atriplex nummularia* L. Plant Physiol **99**: 329-337

Chaudhry B, Yasmeen A, Husnain T, Riazuddin S (1999) Mini-scale genomic DNA extraction from cotton. Plant Mol Biol Rep **17**: 1-7

Chilton MD, Currier TC, Farrand SK, Bendich AJ, Gordon MP, Nester EW (1974) *Agrobacterium tumefaciens* DNA and PS8 bacteriophage DNA not detected in crown gall tumors. Proc Natl Acad Sci USA **71**: 3672–3676

Chilton MD, Drummond MH, Merio DJ, Sciaky D, Montoya AL, Gordon MP, Nester EW (1977) Stable incorporation of plasmid DNA into higher plant cells: the molecular basis of crown gall tumorigenesis. Cell **11**: 263-271

Cooper P, Ho THD (1983) Heat shock proteins in maize. Plant Physiol **71**: 215-222

Das M, Chauhan H, Chhibbar A, Haq QMR, Khurana P (2010) High-efficiency transformation and selective tolerance against biotic and abiotic stress in mulberry, *Morus indica* cv. K2, by constitutive and inducible expression of tobacco osmotin. Transgenic Res **20**:231–246

Davey MW, Stals E, Panis B, Keulemans J, Swennen RL (2005) High-throughput determination of malondialdehyde in plant tissue. Anal Biochem **347**:201–207

del Pozo O, Lam E (1998) Caspases and programmed cell death in the hypersensitive response of plants to pathogens. Curr Biol **8**: 1129–1132

Diaz de Leon L, Arcos L, Diaz de Leon JL, Soto H (1980) Protein biosynthesis by *Bouvardia ternifolia* cell cultures adapted to NaCl. In Proceedings of Second International Workshop on Biosaline Research. LaPaz, Mexico, pp 455-460

Dib TA, Monneveux P, Acevedo E, Nachit MM (1994) Evaluation of proline analysis and chlorophyll fluorescence quenching measurements as drought tolerance indicators in durum wheat (*Triticum turgidum* L. var. durum). Euphytica**79**:65–73

Doke N (1997) The oxidative burst: role in signal transduction and plant stress. In JG Scandalios, eds, Oxidative Stress and the Molecular Biology of Antioxidant Defenses. Cold Spring Harbor Laboratory Press, New York, pp 785–813

Duque P (2011) A role for SR proteins in plant stress responses. Plant Signaling & Behavior **6**: 49-54

Edelbaum O, Ilan N, Grafi G, Sher N, Sram Y, Novick D, Tal N, Sela I, Rubinstein M (1990) Two antiviral proteins from tobacco: Purification and characterization by monoclonal antibodies to human β-interferon. Proc Natl Acad Sci USA **87**: 588-592

Elstner EF (1991) Mechanisms of oxygen activation in different compartments of plant cells. In EJ Pell, KL Steffen, eds, Active Oxygen Species, Oxidative Stress, and Plant Metabolism. Am. Soc. Plant Physiologists, Rockville, MD, pp 13–25

Esterbauer H, Cheeseman KH (1990) Determination of aldehydic lipidperoxidation products: malonaldehyde and 4-hydroxynonenal, Methods Enzymol **186**: 407–421

Esterbauer H, Schaur RJ, Zollner H (1991) Chemistry and biochemistry of 4-hydroxynonenal, malonaldehyde, and related aldehydes, Radic Biol Med **11**: 81–128

Evers D, Overney S, Simon P, Greppin H, Hausman JF (1999) Salt tolerance of *Solanum tuberosum* L. overexpressing an heterologous osmotin-like protein. Biol Plant **42**:105–112

Fan L, Zheng S, Wang X (1997) Antisense suppression of phospholipase D retards abscisic acid and ethylene promoted senescence of postharvest *Arabidopsis* leaves. Plant Cell **9**:2183–2196

Farmer EE, Almeras E, Krishnamurthy V (2003) Jasmonates and related oxylipins in plant responses to pathogenesis and herbivory. Plant Biol **6**: 372–378

Fontes RR, Vilela NJ (2003) The current status of Brazilian crops and future opportunities. Acta Hort **607**:135-141

Food and Agriculture Organization (FAO) (2010) Crop Production Statistics. FAOSTAT,http://faostat.fao.org/site/567/DesktopDefault.aspx?PageID=567#ancor/

Foyer CH, Noctor G (1999) Leaves in the dark see the light. Science **284**:599–601

Gisbert C, Rus AM, Bolarin MC, Lopez-Coronado JM, Arrillaga I, Montesinos C, Caro M, Serrano R, Moreno V (2000) The yeast HAL1 gene improves salt tolerance of transgenic tomato. Plant Physiol **123**:393–402

Goel D, Singh AK, Yadav V, Babbar B, Bansal KC (2010) Overexpression of osmotin gene confers tolerance to salt and drought stresses in transgenic tomato (*Solanum lycopersicum* L.). Protoplasma **245**:133–141

Grillo S, Leone A, Xu Y, Tucci M, Francione R, Hasegawa PM, Monti L, Bressan RA (1995) Control of osmotin gene expression by ABA and osmotic stress in vegetative tissues of wild-type and ABA-deficient mutants of tomato. Physiol Plant **93**:498–504

Halliwell B, Gutteridge JMC (1989) Free radicals in biology and medicine. 2nd Edn. Oxford University Press, Oxford, p 543

Hanson AD, Jacobson JV, Zwar JA (1984) Regulated expression of three alcohol dehydrogenase genes in barley aleurone layers. Plant Physiol **75**: 573-581

Hazell P, Oram P, Chaherli N (2001) Managing Droughts in The Low-Rainfall Areas of The Middle East and North Africa. Environment and Production Technology Division. Discussion paper **78**: 1-3

Hegnauer R (1990) *Umbelliferae.* In R Hegnauer, eds, *Chemotaxonomie der Pflanzen,* Vol 9. Birkhäuser, Berlin, pp: 663-714

Henrik W, Kees L (1972) Isolation and characterization of thaumatin I and II. The sweet-tasting protein from *Thaumatococcus daniellii*. Eur J Biochem **31**: 221-225

Heywood VH (1983) Relationship and evolution in the *Daucus carota* complex. Isr J Bot **32**: 51-65

Hu H, Dai M, Yao J, Xiao B, Li X, Zhang Q, Xiong L (2006) Overexpressing a NAM, ATAF, and CUC (NAC) transcription factor enhances drought resistance and salt tolerance in rice. PNAS **103**:12987-12992

Husaini AM, Abdin MZ (2008) Development of transgenic strawberry (*Fragaria* X *ananassa Duch*.) plants tolerant to salt stress. Plant Sci **174**:446–455

Iyar S, Caplan A (1998) Products of Proline Catabolism Can Induce Osmotically Regulated Genes in Rice. Plant Physiol **116**: 203–211

Jung YC, Lee HJ, Yum SS, Soh WY, Cho DY, Auh CK, Lee TK, Soh HC, Kim YS, Lee SC (2005) Drought-inducible—but ABA-independent—thaumatin-like protein from carrot (*Daucus carota* L.) Plant Cell Rep **24**: 366–373

Kariola T, Brader G, Li J, Palva ET (2005) Chlorophyllase 1, a damage control enzyme, affects the balance between defense pathways in plants. Plant Cell **17**:282–294

Kavi Kishor PB, Hong Z, Miao G-H, Hu C-AA, Verma DPS (1995) Overexpression of Δ^1-pyrroline-5-carboxylic acid synthetase increases proline production and confers osmotolerance in transgenic plants. Plant Physiol **108**: 1387–1394

Kenton P, Mur LAJ, Atzorn R, Wasternack C, Draper J *(*1999*)* Jasmonic acid accumulation in tobacco hypersensitive response lesions. Mol Plant Microbe Interact **12:** 74–*78*

Khanna-Chopra R, Selote DS (2007) Acclimation to drought stress generates oxidative stress tolerance in drought resistant than -susceptible wheat cultivar under field conditions. Environ Exp Bot **60**:276–283

King GJ, Turner VA, Hussey CE, Wurtele ES, Lee SM (1988) Isolation and characterization of a tomato cDNA clone which codes for a salt-induced protein. Plant Mol Biol **10**: 401-412

Kononowicz AK, Nelson DA, Singh NK, Hasegawa PM, Bressan RA (1992) Regulation of Osmotin gene promoter. Plant Cell **4**:513–524

Krishnamurthy R (1991) Amelioration of salinity effect in salt tolerant rice (*Oryza sativa* L.) by foliar application of putrescine. Plant Cell Physiol **32**: 699–703

LaRosa PC, Singh NK, Hasegawa PM, Bressan RA (1989) Stable NaCl tolerance of tobacco cells is associated with enhanced accumulation of osmotin. Plant Physiol **91**:855–861

La Rosa PC, Chen Z, Nelson DE, Singh NK, Hasegawa PM, Bressan RA (1992) Osmotin gene expression is post-transcriptionally regulated. Plant Physiol **100**:409–415

Lauriano JA, Lidon FC, Carvalho CA, Campos PS, Matos MD (2000) Drought effects on membrane lipids and photosynthetic activity in different peanut cultivars. Photosynthetica **38**: 7–12

Li J, Pandeya D, Nath K, Zulfugarov IS, Yoo SC, Zhang H, Yoo JH, Cho SH, Koh HJ, Kim DS, Seo HS, Kang BC, Lee CH, Paek NC (2010) ZEBRA NECROSIS, a thylakoid-bound protein, is critical for the photoprotection of developing chloroplasts during early leaf development. The Plant Journal **62**: 713–725

Liu D, Raghothama KG, Hasegawa PM, Bressan RA (1994) Osmotin overexpression in potato delays development of disease symptoms. Proc Natl Acad Sci USA **91**:1888–1892

Munne-Bosch S, Jubany-Mari T, Alegred L (2001) Drought induced senescence is characterized by a loss of antioxidant defenses in chloroplasts. Plant Cell Environ **24**: 1319–1327

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol Plant **15**: 473-497

Neale AD, Wahleithner JA, Lund M, Bonnett HT, Kelly A, Meeks-Wagner DR, Peacock WJ, Dennis ES (1990) Chitinase, β-1,3-glucanase, osmotin, and extensin are expressed in tobacco explants during flower formation. Plant Cell **2**: 673-684

Nelson D, Raghothama KG, Singh NK, Hasegawa PM, Bressan RA (1992) Analysis of structure and transcriptional activation of an osmotin gene. Plant Mol Biol **19**:577–588

Noori SAS, Sokhansanj A (2008) Wheat plants containing an osmotin gene show enhanced ability to produce roots at high NaCl concentration. Russ J Plant Physiol **55**:256–258

Ober ES, Bloa ML, Clark CJA, Royal A, Jaggard KW, Pidgeon JD (2005) Evaluation of physiological traits as indirect selection criteria for drought tolerance in sugar beet. Field Crops Res **91**:231–249

Oya T, Nepomuceno AL, Neumaier N, Farias JRB, Tobita S, Ito O (2004) Drought tolerance characteristics of Brazilian soybean cultivars- evaluation and characterization of drought tolerance of various Brazilian soybean cultivars in the field. Plant Prod Sci **7**:129-137

Parkhi V, Kumar V, Sunilkumar G, Campbell LM, Singh NK, Rathore KS (2009) Expression of apoplastically secreted tobacco osmotin in cotton confers drought tolerance. Mol Breed **23**:625–639

Pastori GM, Trippi VS (1992) Oxidative stress induces high rate of glutathione reductase synthesis in a drought-resistant maize strain. Plant Cell Physiology **33**: 957–961

Pawlicki N, Sangwan RS, Sangwan-Norreel (1992) Factors influencing the *Agrobacterium tumefaciens*-mediated transformation of carrot (*Daucus carota* L.) Plant Cell, tissue and organ culture **31**: 129-139

Peterson CE, Simon PW (1986) Carrot Breeding. In MJ Bassett, eds, Breeding Vegetable Crops. AVI Publishing, Westport

Pierpoint WS, Jackson PJ, Evans RM (1990) The presence of a thaumatin-like protein, a chitinase and a glucanase among the pathogenesis-related proteins of potato (*Solanum tuberosum*). Physiol Mol Plant Pathol **36**: 325-338

Pollard A, Wyn Jones RG (1979) Enzyme activities in concentrated solutions of glycinebetaine and other solutes. Planta **144**: 291–298

Quan RD, Shang M, Zhang H (2004) Improved chilling tolerance by transformation with betA gene for the enhancement of glycinebetaine synthesis in maize. Plant Sci 166:141–149

Raghothama KG, Liu D, Nelson DE, Hasegawa PM, Bressan RA (1993) Analysis of an osmotically regulated pathogenesis-related osmotin gene promoter. Plant Mol Biol **23**:1117–1128

Rajendrakumar CSV, Reddy BVD, Reddy AR (1994) Proline protein interactions: protection of structural and functional integrity of M4 lactate dehydrogenase. Biochem Biophys Res Commun **201**: 957–963

Ramanjulu S, Sudhakar C (2000) Proline metabolism during dehydration in two mulberry genotypes with contrasting drought tolerance. J Plant Physiol **157**:81–85

Rathore KS, Sunilkumar G, Campbell LM (2006) Cotton (*Gossypium hirsutum* L.). In K Wang, eds, Methods in molecular biology: *Agrobacterium* protocols, Ed 2 Vol 1. Humana Press Inc, Totowa, pp 267–279

Restrepo MA, Freed DD, Carrington JC (1990) Nuclear transport of plant potyviral proteins. Plant Cell **2**:987–998

Richardson M, Valdes-Rodriguez S, Blanco-Labra A (1987) A possible function for thaumatin and a TMV-induced protein suggested by homology to a maize inhibitor. Nature **327**: 432-434

Ringel C, Siebert S, Wienhaus O (2003) Photometric determination of proline in quartz microplates: remarks on specificity. Anal Biochem **313**:167–169

Rubatzky VE, Quiros CF, Simon PW (1999) Carrots and related vegetable *Umbelliferae.* CABI Publ., New York

Sachs MM, Freeling M, Okimoto R (1980) The anaerobic proteins of maize. Cell **20**: 761-767

Saenz LC (1981) Research on *Daucus* L. (*Umbelliferae*). Aneles Jardin BotanicaDe Madrid **37**: 481-533

Sairam RK, Deshmukh PS, Saxena DC (1998) Role of antioxidant systems in wheat genotypes tolerant to water stress. Biol Plant **41**: 387-394

Scarpari LM, Meinhardt LW, Mazzafera P, Pomella AWV, Schiavinato MA, Cascardo JCM, Pereira GAG (2005) Biochemical changes during the development of witches` broom: The most important disease of cocoa in Brazil caused by *Crinipellis perniciosa*. J Exp Bot **56**: 865-877

Schonfeld MA, Johnson RC, Carver BF, Mornhinweg DW (1988) Water relations in winter wheat as drought resistance indicators. Crop Sci **28**:526–553

Shulaev V, Oliver DJ (2006) Virginia bioinformatics metabolic and proteomic markers for oxidative stress. New tools for reactive oxygen species research. Plant Physiol **141**:367– 372

Simon PW (1993) Carrot, *Daucus carota* L. In G Kaloo, B Bergh, eds, Genetic Improvement of Vegetable Crops, Pergamon Press Ltd., Tarrytown, NY.

Simon PW (1997) Plant pigments for color and nutrition. HortScience **32**: 12-13

Simon PW (2000) Domestication, historical development, and modern breeding of carrot. Plant Breed Rev **19**:157-190

Simpson KL (1983) Relative value of cartenoids as precursors of vitamin A. Proc Nutr Soc **42**: 7-17

Sinclair TR, Ludlow MM (1985) Who taught plants thermodynamics? The unfulfilled potential of plant water potential. Aust J Plant Physiol **12**:213–217

Singh NK, Bracker CA, Hasegawa PM, Handa AK, Buckel K, Hermodson MA, Pfankoch E, Regnier FE, Bressan RA (1987) Characterization of osmotin: a thaumatin-like protein associated with osmotic adaptation in plant cells. Plant Physiol **85**:529–536

Singh NK, Handa AK, Hasegawa PM, Bressan RA (1985) Proteins associated with adaptation of cultured tobacco cells to NaCl. Plant Physiol **79**:126–137

Singh NK, Nelson DE, Kuhn D, Hasegawa PM, Bressan RA (1989) Molecular cloning of osmotin and regulation of its expression by ABA and adaptation to low water potential. Plant Physiol **90**:1096–1101

Sivakumar MVK, Wilhite DA, Motha RP, Wood DA (2011) Agricultural Drought Indices, Proceedings of an Expert Meeting. World Meteorological Organization, AGM-11

Smirnoff N, Cumbes QJ (1989) Hydroxyl radical scavenging activity of compatible solutes. Phytochemistry **28**: 1057–1060

Smirnoff N (1998) Plant resistance to environmental stress. Curr Opin Biotechnol **9**:214–219

Song NH, Ahn YJ (2010) DcHsp17.7, a small heat shock protein from carrot, is upregulated under cold stress and enhances cold tolerance by functioning as a molecular chaperone. HortScience **45**:469-474

Sreenivasulu N, Grimm B, Wobus U, Weshke W (2000) Differential response of antioxidant compounds to salinity stress in salt-tolerant and salt-sensitive seedlings of foxtail millet (*Setaria italica*). Physiol Plant **109**: 435–442

Stavric B (1994) Role of chemopreventers in human diet. Clinical Biochemistry **27**: 319-332

Stewart GR, Larhar F (1980) Proline. In PK Stumpf, EE Conn, eds, The Biochemistary of Plants, Vol 5. Acadimic press, New York, pp 609-635

Stintzi A, Heitz TS, Kauffmann S, Legrand M, Fritig B (1991) Identification of a basic pathogenesis-related, thaumatin-like protein of virus-infected tobacco osmotin. Physiol Mol Plant Pathol **38**:137–146

Stupnikova IV, Borovskii GB, Dorofeev NV, Peshkova AA, Voinikov VK (2002) Accumulation and disappearance of dehydrins and sugars depending on freezing tolerance of winter wheat plants at different developmental phases. J Therm Biol **27**:55–60

Sung ZR, Okimoto R (1981) Embryonic proteins in somatic embryos of carrot. Proc Natl Acad Sci **78**: 3683-3687

Sunilkumar G, Campbell LM, Waghela SD, Rathore KS (2009) Expression of anti-K99 scFv in rice tissues and its functional characterization. Trans Res **18**:347-360

Sunilkumar G, Rathore KS (2001) Transgenic cotton: factors influencing *Agrobacterium*-mediated transformation and regeneration. Mol Breed **8**:37–52

Thomas JC, DeArmond RL, Bohnert HJ (1992) influence of NaCl on growth, proline, and phosphoenolpyruvate carboxylase levels in *Mesembryaathenum crystallinum* suspension cultures. Plant physiol **98**: 174-182

United Nations Development Programme (UNDP) (2011) Drought Impact Assessment, Recovery and Mitigation Framework and Regional Project Design in Kurdistan Region (KR), pp: 5-6

Vigers AJ, Wiedemann S, Roberts WK, Legrand M, Selitrennikoff CP, Fritig B (1992) Thaumatin like pathogenesis-related proteins are antifungal. Plant Sci **83**:155–161

Vilela NJ (2004) Cenoura: um alimento nobre na mesa popular. Horticultura Brasileira 22: cover article.

Vinocur B, Altman A (2005) Recent advances in engineering plant tolerance to abiotic stress: achievements and limitations. Curr Opin Biotechnol **16**:123–132

Vollenweider S, Weber H, Stolz S, Chetelat A, Farmer EE (2000) Fatty acid ketodienes and fatty acid ketotrienes: Michael addition acceptors that accumulate in wounded and diseased *Arabidopsis* leaves, Plant J. **24**: 467–476

Wanner L, Junttila O (1999) Cold-induced freezing tolerance in *Arabidopsis*. Plant physiol **120**: 391-399

Weber H, Chételat A, Reymond P, Farmer EE (2004) Selective and powerful stress gene expression in *Arabidopsis* in response to malondialdehyde, Plant J. **37**: 877–889

Wilhite DA, Buchanan-Smith M (2005) Drought as hazard: Understanding the natural and social context. In DA Wilhite, eds, Drought and Water Crises: Science, Technology, and Management Issues. CRC Press, Boca Raton, Florida

Wilhite DA, Glantz MH (1985) Understanding the drought phenomenon: The role of definitions. Water International **10**:111-120

Woloshuk CP, Meulenhoff SJ, Sela-Buurlage M, van den Elzen PJ, Cornelissen BJ (1991) Pathogen-induced proteins with inhibitory activity toward *Phytophthora infestans*. Plant Cell **3**:619–628

Yamada M, Morishita H, Urano K, Shiozaki N, Yamaguchi-Shinozaki K, Shinozaki K, Yoshiba Y (2005) Effects of free proline accumulation in petunias under drought stress. J Exp Bot **56**:1975–1981

Zhu B, Chen THH, Li PH (1993) Expression of an ABA responsive osmotin-like gene during the induction of freezing tolerance in *Solanum commersonii*. Plant Mol Biol **21**:729–735

Zhu B, Chen THH, Li PH (1995) Expression of three osmotin-like protein genes in response to osmotic stress and fungal infection in potato. Plant Mol Biol **28**:17–26

Zlatev ZS, Lindon FC, Ramalho JC, Yordanov T (2006) Comparison of resistance to drought of three bean cultivars. Biol Plant **50**:389–394